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MOLECULAR STRUCTURE IN PROTOPLASM

By

O. L. SPONSLER

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This opportunity to discuss the structure of protoplasm with a group of specialists in closely related fields of thought is indeed a great pleasure. It is an opportunity of distinctly peculiar value, for if there is one thing certain about the conception of protoplasmic structure it is that the conception is a resultant arrived at through the work of many specialists in varied lines of investigation. Although various ideas concerning the structure have appeared in the literature for the past hundred years, a conception of the molecular aspect has had to await the modern developments in molecular physics, until atoms and molecules began to assume actuality. The trend of thought along this line became apparent when it was shown that the cellulose of the cell wall of the plant consisted of long indefinite molecular chains of glucose residues (1). At that time, 1925, the impression arose that similar chain structures might occur in the organized protoplasm, and through daily contact with concepts of glucose molecules as three-dimensional entities, a feeling of reality was engendered in the spatial nature of the constituent molecular structures in the protoplasmic matrix. Several years later when it was shown by x-ray analyses that the proteins must be considered physically as chains of amino acid residues (2, 3 and 4) just as cellulose was known to consist of chains of glucose residues, a molecular concept of protoplasm became feasible. The concept was rather vague and indistinct, it is true, but nevertheless a structure on the molecular level began to appear. The feeling for structure became more and more evident, and five or six years ago when several of my students became interested we began to think seriously of protoplasm from a molecular point of view.

The complexities and variables, which are always encountered in working with living materials, left us at first with the impression that an attempt to gain a molecular understanding of protoplasm was hopeless; but we could not quite bring ourselves to believe that there was no possible way out of the situation; and I think this stubbornness was partially rewarded as we gradually realized that by simplification of concepts we might find a road to follow. We have employed this simplifying process repeatedly as a means of developing concepts and of bringing them within our grasp. Many of these were found to serve our purposes admirably as a means of developing further basic ideas; some, however, have had to be altered to fit newer viewpoints. It occurred to us also that we would be able to comprehend protoplasmic

interrelations more clearly if at first we built up static structures, or frameworks on which to hang more active mechanisms, somewhat as in microscopic methods in cytology; and later tried to develop the dynamic aspects.

In attempting to find a point of attack we searched for microscropic structures in protoplasm which would be suitable for x-ray methods of structural study and which could be considered at the same time as unmodified protoplasm. Crystals of proteins and other substances were available, but seemed unsuitable since they had lost most of the characteristics of protoplasm. Such things as hair, feathers, horn, keratins in general, muscle fibers, nerve fibers, etc., many structures of animal but few of plant origin, were available and fairly suitable for x-ray studies; these, however, are so specialized that they could give us little more than possible indications of what protoplasm could do in building structures out of proteins, just as we had already found what it could do in building starch grains and cellulose walls from glucose chains. Direct means, however, of molecular approach to cytoplasm in the natural state seemed to be impossible, and it became more and more evident that any advance in molecular structural studies would have to be made by correlation of bits of indirect evidence obtained from many fields of investigation. This meant that we would have to proceed step by step and verify and check every step until reasonable certainty was obtained for each point, since each advance must depend upon the preceding steps. This meant also that we would have the doubtfully delightful, but enormous task of searching the literature in many lines of investigation in physics, chemistry and biology, and of interpreting these experimental results in the light of a molecular protoplasmic viewpoint.

We soon found ourselves involved in conceptions which seemed to be in opposition to apparently simple commonplace points of view. Conflicting ideas began to develop when conceptions from one dimensional level or from one field of experimental procedure were carried to another. The biological point of view goes counter to that of the physical-chemical in many ways; and perhaps the most obvious contrast is found when the consequences resulting from orderliness of parts is opposed to random distribution. These contrasts between the physical and the biological sciences become so fundamental in their nature that it is highly important to keep the two viewpoints distinctly separated.

The foundations of parts of physics and most of chemistry are based on a statistical world where chance and probability come into play to furnish certain laws of order which are garnered from random motion and random distribution. On quite the other hand, the foundations of biology are based, to a remarkable degree, on the individual where orderliness and structure prevail. A simple illustration is found in the

orderly behavior of the chromosomes at mitosis as contrasted with the behavior which should occur under the purely physical conception of diffusion.

Throughout a discussion of this sort it is always necessary to keep in mind that several dimensional levels are constantly under consideration; among these the three most commonly recognized are, the molecular level, the sub-microscopic or micellar range, and the microscopic level. A substance which is considered particulate on one level may be considered as continuous on another; a fluid on one, may be definitely structural on another; order on one, may be chaos on another; and so on for many oppositions, such as those included in inert and living, inorganic and organic, heterogeneous and homogeneous; thermal random movement as opposed to orderly movement in mechanisms; and especially, the relation of sizes on one level in contrast to sizes on another.

In our attempts to obtain a comprehension of a structural picture based on molecular structures we started with what we are calling here the smallest of particles, the atoms; and in a sense built them into larger particles, the molecules. These were then built into aggregates in the colloidal, micellar or sub-microscopic range. This brought the materials almost within reach of the microscope. In the lower levels the only approach was from a physical-chemical viewpoint; in the micellar levels the biological viewpoint began to encroach upon the physical; while on the microscopic level physical-chemical methods became only occasional tools to aid in a biological understanding. In other words a random situation of lower levels became more and more orderly as we approached the higher levels of biological structures.

Our first step was a study of the composition of protoplasm. Analyses of protoplasm from many organisms were found to vary considerably due to lack of standards of both the methods of analyses and of the condition of organisms. But despite these inconformities a set of values were chosen which seems reasonably representative of minimum amounts; these are presented in Table I.

TABLE I
COMPONENTS OF PROTOPLASM

Component	Range of Proportions	Generalized Proportions
Water	65 to 90%	90%
Proteins	6 to 20%	7%
Fatty substances	1 to 13%	1%
Other organic materials	0 to 16%	1%
Inorganic materials	1 to 4%	1%

From such analyses we may gain several things. There is considerable likeness in composition of the various protoplasms, which we have generalized in the third column, on an assumption that probably all protoplasms when consisting of 90% water will contain at least 7% protein, 1% fatty substances, 1% various other organic substances and 1% inorganic materials. These are basic quantities which we have simplified by making use of the generic terms, fatty substances, inorganic materials, etc.

The table may be used for determination of approximate amounts of the variously sized molecules. The proteins are large molecules, perhaps of molecular weight 35,000. The fatty substances have smaller molecules, although still large. Lecithin, cholesterol, oils in general, have molecuar weight of, say, 1,000. Other organic molecules, hexoses, smaller oganic acids, etc. may average about 200; while the inorganic material will have an average molecular weight of perhaps 50 to 75. From these averages we may compute the proportional number of molecules of each sort and gain a slightly different picture; still, however, only an approximation. Thus in our minimum mixture we will have about 20,000 water molecules which we may assume surround a single protein molecule. In this shell of water there are 5 molecules of fatty substances, 25 smaller organic molecules and about 50 inorganic ions. This gives us a crude impression of one enormous molecule surrounded by many very small molecules and only a few of intermediate size.

When we convert these to volume proportions, the minimal picture remains about the same. The large amount of water, 90% with a density of 1.00, and the next larger amount of proteins with density 1.3 shows that almost 97% of the volume consists of only these two substances. This means that structurally about 97% of the protoplasm consists of only two sorts of molecules; and probably 98% of only three sorts, for the fatty substances are less dense than water. If we are willing to accept these values the way is open for a structural picture, providing there is sufficient information available concerning these two or three types of molecules and concerning their behavior toward one another.

The picture, as it now stands, has volume but no form or structure. If we can obtain some conception of the structure of these three molecules, or even of the two sorts, water and protein, we may be able to put them together and get a preliminary glimpse of the protoplasmic matrix.

To this end we turn to the available information concerning the water molecule, the protein, and also the fatty substances. The amount and extent of this information is perhaps well known to you; but I think it needs a little critical selection for our use in protoplasm, for unfortunately we cannot take bits of protein and bits of fatty material out of a living cell and study them at leisure without alteration.

A preliminary study of the information available shows that we must deal with the composition of these molecules, and also that we must examine the sources of the materials which have been studied, since many important properties are characteristics of the parts rather than of the whole molecule.

The molecules of the proteins and of the fatty materials are, as mentioned, very large, and for that reason are not suitable for study in great atomic detail by present day experimental methods. It is necessary therefore to bring evidence of various sorts to bear upon each individual feature of structure in order to obtain reasonably certain conclusions. If the position of every atom in the molecule were determinable, confirmatory evidence would be a superfluity. But these positions are not known by direct physical experiment, and therefore we have taken considerable pains to determine by other means the relative positions of the various parts of the molecules, and also to determine their size and form. The effort which we made along these and similar lines were in some instances fairly direct; in some, rather roundabout.

It soon became evident that we would have to draw rather definite boundary lines, for a truthful picture of active protoplasm is analogous to a long roll of movie film with a thousand consecutive frames. We felt that we must for this reason restrict ourselves to some kind of static picture, that is, to one frame out of the movie roll, and deal with the dynamic states specifically as occasion arose. In employing the static conception of the molecule we could make use of models rather easily and modify these wherever kinetic conceptions were desired. For clarification in carrying our ideas from one level of spatial dimensions to another we found enlargement up to our every day level of existence a great convenience. This often brought out crude comparisons which, however, were helpful in forming these conceptions.

Since, as shown in Table I, 90% of the active protoplasm is water and 7% protein, it is evident that we would have to form definite conceptions of the water molecule and of the protein molecule; and further, to determine their relationships to one another. The structures of these two molecules, we assumed, would allow us to make a fairly satisfactory structural framework upon which to hang the necessary fatty molecules as well as the ions and molecules of smaller size.

The water molecule of only three atoms seemed at first to be a simple structure to deal with, but we found this simplicity to be a snare and a delusion. The protein molecule with its thousand atoms seemed an impossible task to model, but curiously it became relatively simple. The structural importance of the fatty molecules comes into view more perhaps when dealing with specific situations than when considering the more general aspect of the protoplasmic matrix.

Upon examining the atomic components it was realized that there were only four sorts of atoms to be considered, except in occasional specific instances. These are shown in Table II where the percentage of the materials gives one an approximation of the large amount of only four sorts of atoms. The four atoms, C, H, O and N, make up fully 98 or 99% of the living material; the others, so far as volume is concerned, are unimportant.

TABLE II
ATOMIC COMPONENTS OF PROTOPLASMIC CONSTITUENTS

Constituent	Amount Present	Atomic Components
Water	90%	H, O
Protein	7%	C, H, O, N (S)
Fatty materials	1%	C, H, O, (N) (P)
Other organic matter	1%	C, H, O

Only two characteristics of the atoms are necessary in order to use them as building units; one is the radius of the atom; the other is the directive effect of its primary valence bonds. These are shown in Table III where the number of primary valence bonds for each atom is given along with the radii for single, double and triple bonds, and the directive angles. This set of working dimensions is well established through

TABLE III
BONDS, RADII AND DIRECTIONAL ANGLES

	H	O	N	C
Number of single bonds	1	2	3	4
Angle for single bonds		105°	106°	109.5°
Radii for single bonds	0.29Å	0.66Å	0.70Å	0.77Å
Angles for one double bond			110°	125°
Radii for double bonds		0.58Å	0.63Å	0.69Å
Radii for triple bonds			0.55Å	0.61Å

experimental and theoretical studies, and may be found in several recent books (5, 6, 7 and 8). The values converted into graphical structures are shown in the diagrams in Figure 1 where the circle represents a central portion of the atom; and the lines indicate the single bonds placed more or less at the proper angles. In the lower half of the figure examples of combinations are given showing the sums of the radii. With the aid of these two properties of the atom, the formulae determined by the chemist may now be made into three-dimensional structures, since he has been able in most cases to tell which atoms are

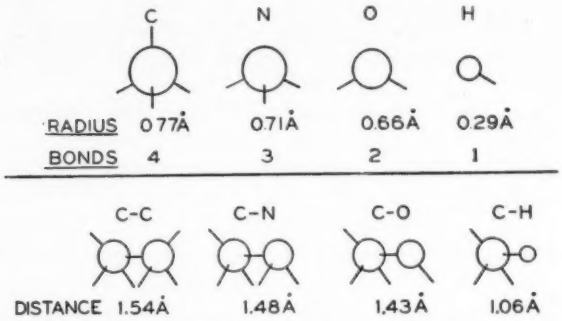


Fig. 1. Diagrams to illustrate bond angles and atomic radii of the four atoms, carbon, nitrogen, oxygen and hydrogen, above; combinations of these with additive interatomic distances are shown below the line.

attached to which. The chemist has shown, in literally hundreds of examples, that the x-ray crystal structures are correct; or, depending upon your prejudices, the x-ray crystal structure physicist has shown the chemist to be right. One may now diagrammatically take the oxygen atom and attach two hydrogens to it at about 109° apart (105° may be more exact) and have a model of a water molecule which fits the behavior of water (21); or one may build a glycine molecule by attaching the appropriate number of C, O, H, N, atoms together according to the chemist's formula and find that it fits the x-ray determinations of volume and of atomic positions (40). These interrelations, in fact, have become so well established that one need not hesitate to build a model of any organic molecule and feel that, dimensionally, it will be about as accurate as the chemical data will allow. I shall not dwell further upon the many, many illustrative examples which are available but, instead, shall accept these values as experimentally verified and make use of them in a study of the water and the protein molecules.

Several viewpoints and a number of protoplasmic and atomic concepts have been introduced which may be generally unfamiliar, and for that reason, a brief summary may be worth while. The generalized conception of protoplasm as consisting of 90% water, 7% protein and the remaining 3% consisting of fatty substances and other organic and inorganic materials, is perhaps unusual. However, it allows a first approach to be made to a molecular picture, since the water molecules are very small and therefore many of them are contained in the 90% portion, while the protein molecules are very large with relatively very few in the 7% portion. Thus the generalized picture shows, as mentioned previously, one protein molecule accompanied by 20,000 water molecules, 5 fatty and 25 smaller organic molecules and by about 50 or more inorganic ions. In order to obtain a structural picture, dimensional values are needed, and to this end the radii and directive valence

angles are given for the few types of atoms which constitute about 98% or more of the protoplasmic mass. These few points are the basic facts, the data, upon which we have relied for most of our structural work. With these points in mind we turn to the stuctural features of the three important molecules and their interrelations.

Several features of the proteins seem to be well established. Let me hedge a little by modifying that in this way—well enough established to be employed in dimensional studies of not too specific nature. These features refer to composition and to spatial dimensions. We may accept the protein as being composed almost, if not entirely, of amino acid residues. In Table IV a number of protein analyses are given merely to show the occurrence of these various amino acid constituents.

TABLE IV
AMINO ACID COMPOSITION OF VARIOUS PROTEINS

Amino Acid	Composition in percent of dry weight				
	Edestin (47)	Zein (47)	Gelatin (48)	Gliadin (48)	Insulin (49)
Glycine	3.8	0.0	25.5	0.5	—
Alanine	3.6	9.8	8.7	2.0	—
Valine	6.3	1.9	0.0	3.3	—
Leucine and Iso-leucine	14.5	25.0	7.1	6.6	30.0
Phenylalanine	3.1	7.6	1.2	2.4	—
Tyrosine	4.6	5.9	0.01	3.3	12.2
Tryptophane	2.5	0.2	0.0	1.1	—
Glutamic acid	19.2	31.3	5.8	43.0	30.0
β-Hydroxyglutamic	—	2.5	0.0	7.7	—
Aspartic acid	10.2	1.8	3.4	0.8	—
Proline	4.1	9.0	19.4	10.3	—
Hydroxyproline	—	—	14.4	—	—
Serine	0.3	1.0	0.4	0.2	—
Hydroxyvaline	—	1.5	—	—	—
Cystine	1.0	0.8	0.2	2.1	12.6
Methionine	2.1	2.3	1.0	2.0	—
Arginine	15.8	1.8	9.1	2.9	3.2
Histidine	2.2	0.8	0.9	1.5	8.0
Lysine	2.2	0.0	5.9	0.6	2.3
Ammonia	2.3	3.6	0.4	5.2	1.65
Total	97.9	106.8	103.4	95.5	99.95

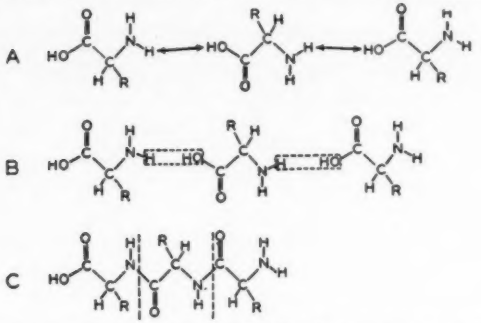


Fig. 2. Diagrams to show formation of peptide linkage by the splitting out of water between amino acids. A, shows three separate amino acids; B, the source of the H and OH; C, the linkage after the water molecule has been split off.

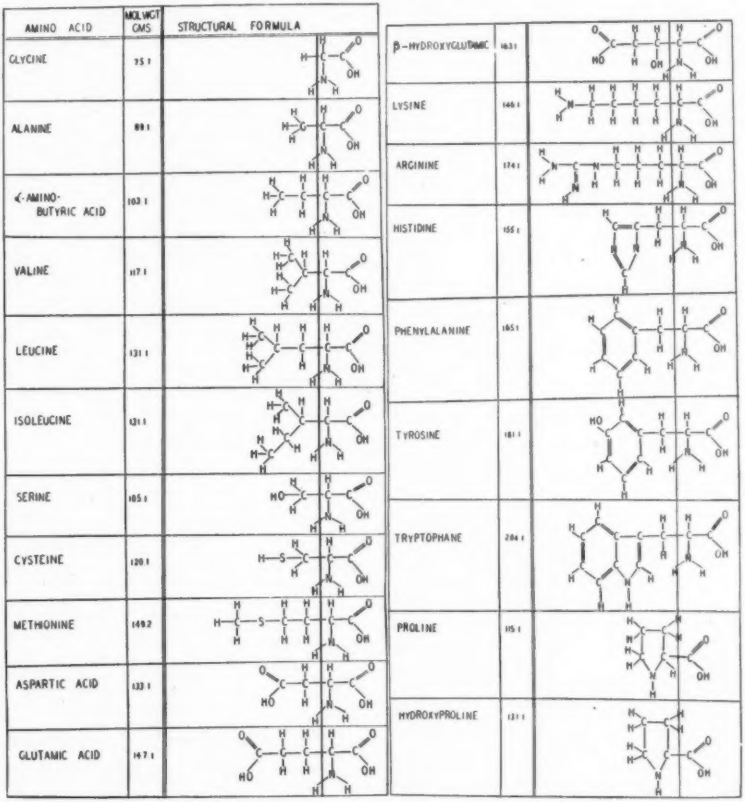


Fig. 3. Diagrams of amino acid formulae. The feature common to all is shown on the right of the vertical line; on the left, the residues which give the amino acids their characteristic properties are shown.

A second feature is that the amino acid residues are, almost without exception, attached to one another through peptide linkages as indicated in the formulae in Figure 2 where several stages of the formation of a peptide linkage are shown.

A third feature lies in the curious fact that the generic characterizing structure of the amino acid, that is, the COOH and NH_2 groups, are attached to the alpha carbon in the same spatial arrangement in all *natural* proteins. This generic feature is indicated in Figure 3 where the COOH and NH_2 groups are shown on the right hand side of the vertical line.

The peculiar consequence of this likeness between amino acids is brought out in Figure 4 where the peptide attachment results in a chain with the residues extending alternately on the opposite sides of a zigzag backbone. This is in agreement with x-ray data and indicates a

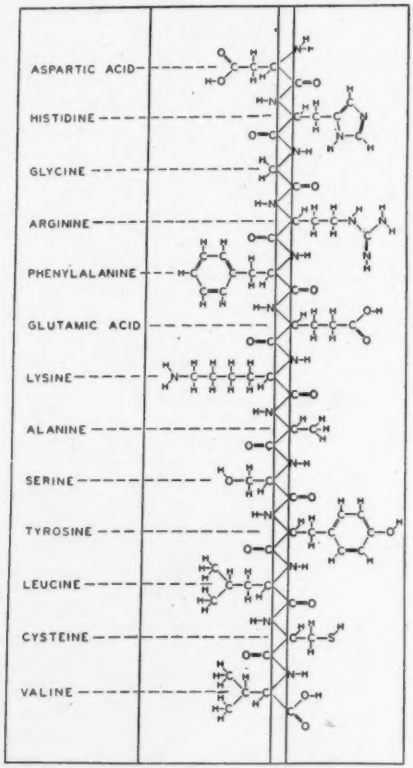


Fig. 4. Diagram showing the long extended backbone formed by the peptide linkage between the amino acid residues.

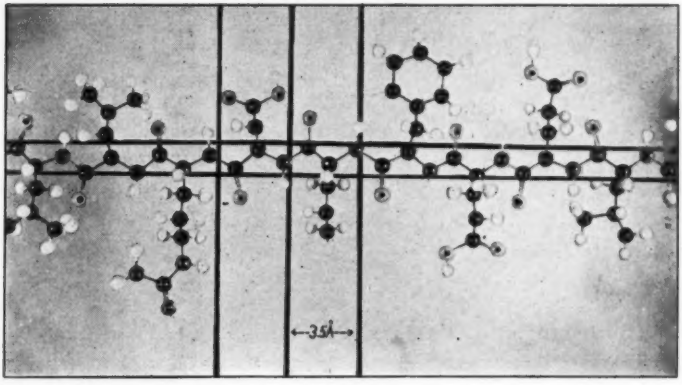


Fig. 5. Model of a portion of a protein chain made to scale using known interatomic distances and bond angles. The large, black spheres represent carbon atoms; the small white ones, hydrogen atoms. Those marked with a black dot represent oxygen atoms; and those with a cross, nitrogen atoms.

structure which may be represented by the three-dimensional model of Figure 5, where the spheres represent the positions of the various atoms made to a very much enlarged scale.

As a result of these structural features the spatial dimensions of thickness and width of the polypeptide chain are more or less fixed at about 4.5Å and 10.5Å even for proteins of rather specialized nature. These values are in good agreement both experimentally and theoretically. As one example of this agreement, Table V gives these values

TABLE V
BACKBONE AND SIDE CHAIN SPACINGS IN VARIOUS PROTEINS

Protein	Backbone Spacing (Å)	Side-Chain Spacing (Å)	Reference
Pepsin	4.5	11.5	9
Tobacco seed globulin	4.4	11.0	9
Squash seed globulin	4.4	11.0	9
Edestin	4.5	11.0	9
Egg albumin	4.7	10.0	10
	4.7	10.6	9
Zein (corn)	4.6	9.8	9
Fibrin	4.7	10.1	11
Collagen	4.6	10.0	12, 13
Keratin (hair)	4.7	9.8	14, 15
Cytoplasmic protein (cabbage)	4.4	9.6	16
Serum albumin	4.4	9.6	9
Casein (milk)	4.5	10.2	9

for a few of many proteins as determined by many investigators using the x-ray method of structural analysis.

In the chain each residue has a definite portion of the backbone allotted to it. This may be seen in Figure 5. The distance for each residue is about 3.5Å, varying slightly with the protein. The total length of the chain thus depends upon the number of amino acid residues of which it is composed. If it consists of about 300, the chain will be approximately 1000Å long, regardless of whether it is fully extended, rolled up into a ball, or folded into a monolayer or into a globular particle of indefinite shape. Figure 6 shows a model of a protein chain corresponding to approximately one-fifteenth of a chain of about 1000Å in length.

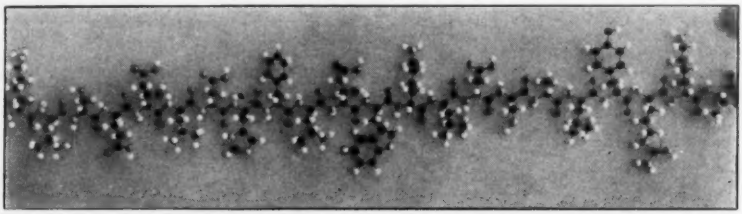


Fig. 6. Model of a longer section of a protein chain similar to that shown in Fig. 5.

In a similar additive manner, if the percentage composition of the component amino acid residues is known, the molecular weight of the chain may be computed. Thus three characteristics of the chain, found experimentally—spatial dimensions, number of residues, and weight all become interrelated; for example, a chain of 300 residues having an average residue weight of 120 will have a molecular weight of 36,000 and will be about 1000Å in length. Also it will have a thickness of about 4.5Å and a width of about 10.5Å. These values have been checked by many experimental methods and by theory and computation. We shall return again to these farther on.

The fatty materials in which we are most interested have not yet been studied structurally with the same intensity as the proteins. The two most prominent and also most universal in protoplasm are the phospholipides, such as lecithin, and the sterols, such as cholesterol. To obtain the structure and dimensions of lecithin we have combined the chemical knowledge (17 and 18) with the atomic dimensions, since aside from the dimensions of the aliphatic chain, the dimensions for the molecule as a whole have not been determined. A model furnishes the picture as shown in Figure 7.

The second form of fatty substances, the sterols, has received more attention structurally (19 and 20), and of these cholesterol is perhaps the most important from the spatial structure point of view in proto-

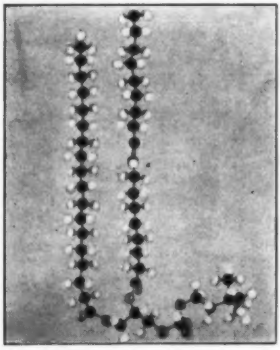


Fig. 7. Molecular model of lecithin. Made to scale using known interatomic distances and bond angles.

plasm. Its molecule is more compact on account of its ring structures than that of lecithin, as may be seen from the model shown in Figure 8.

The third type of molecule, that of water, while the smallest and by far the most frequent in occurrence in protoplasm is by no means as simple as its three atom composition might lead one to suppose. Although it consists of three atoms, the two hydrogens are so small that the molecule as a whole is no larger than an individual atom; and as a molecule it is one of the smallest. In Figure 9 a diagram shows the relation of the hydrogens to the central oxygen atom.

The water molecule possesses a number of properties which are intimately associated with the structural features of protoplasm as well as with many vital activities. To comprehend these we must know its structure in considerable detail. This has been discussed intensively (21) in the literature and needs, perhaps, only a slight reference to the diagram in Figure 10. Attention is directed to the residual charges,

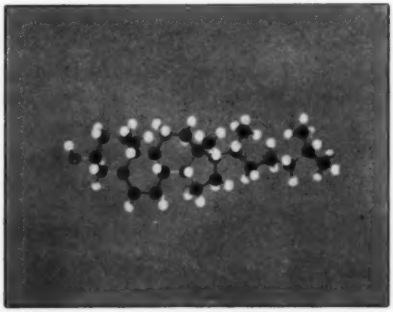


Fig. 8. Model of cholesterol. Large black balls represent carbon atoms; large gray ball, oxygen atom; and small white balls, hydrogen atoms (20).

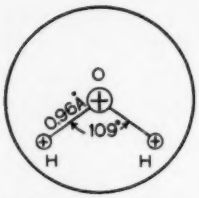


Fig. 9. Planar diagram through the center of the water molecule showing the placement of the oxygen and hydrogen nuclei (21).

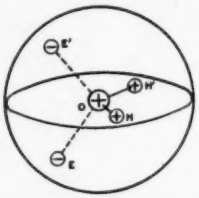


Fig. 10. Three-dimensional diagram of a water molecule showing the tetrahedral arrangement of the two hydrogen nuclei and the two residual negative charges, around the central oxygen nucleus (21).

two positive and two negative, which occur at tetrahedral points in the molecule. Through the directive nature of the bonding electrons of the oxygen atom the hydrogens take the positions as shown. Two residual negative charges are concentrated at positions which form with the hydrogens the four corners of a tetrahedron. These several features make the water molecule a structure which is especially versatile in the production of ions, and in the formation of hydrogen bridges and polymers, as well as hydration layers. Its electron-proton system is rather weakly stable, and, as a result, the looseness with which the hydrogen protons are held in place facilitates both hydrogen ion and hydrogen bridge formation. The water molecule thus becomes of great importance in protoplasmic structures and activities.

Molecules of these three types; water, protein and fatty substances, furnish about 98% of the building units in protoplasm; and since the atoms of each molecule are held intact by primary valence forces, we may think of these molecules as structural entities so long as primary valence and the short distances, about 1.5Å associated with them, are under consideration. Structural relations between these molecular entities involve, however, weaker forces than those of primary valence, and correspondingly greater distances. Three types of weaker forces are more or less distinct; namely, ionic forces, between oppositely charged atoms with a distance of about 2.5Å as closest approach; hydrogen bridge bonding, effective between oxygen and nitrogen atoms where the distance is about 2.8Å as shown in the diagram in Figure 11;

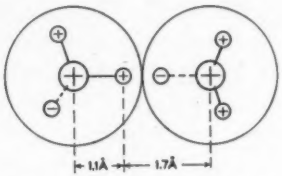


Fig. 11. Diagram of two water molecules showing the formation of a hydrogen bridge between them. The small circle containing a plus charge represents a hydrogen nucleus or proton being attracted to the residual negative charge ⊖ on the adjacent molecule (21).

and van der Waals' forces effective between all molecules in bringing them to a distance of about 3.5 to 4Å from one another. This is the weakest of the three means of cohesion.

Consideration of the structural relations involving the molecules of proteins, water and fats; the cohesion forces just mentioned, ionic, hydrogen bridge and van der Waals', and the proportional number of the molecules, brings us a long ways towards an understanding of the structures, mechanisms and activities in the protoplasmic matrix. To illustrate these inter-relations I shall refer first to only one type of protein chain configuration, one which seems to be the simplest for the purpose, that is, the unfolded or extended chain. A model of a short section of such a chain is shown in Figure 6. *Where* it occurs in nature and *whether* it ever occurs in the extended state is, for the moment, unimportant.

As a first attempt, if we imagine a mixture consisting of 10% of these chains and 90% water molecules and think of a uniform distribution, then each chain will be surrounded by its allotment of 20,000 water molecules. The chain may be 4.5Å × 10Å × 1000Å long, and the water molecules may be thought of as cubes of 3Å on a side. If all of the 20,000 water molecules were distributed uniformly about the protein chain they would form only 3 surrounding layers; or on the average there would be only 6 layers separating the chains. A vague picture of this sort represents an average condition which merely gives us the feel of dimensions. A more truthful picture would present the chain as consisting of amino acid residues of varying lengths with the water molecules clustered at the polar endings of certain residues; that is, around the residues which have oxygen or nitrogen end groups, such as, aspartic and glutamic acids and serine which have oxygen end groups; and arginine, histidine and lysine, which have nitrogen atoms at the outer end of the residues. It will be recalled that the atoms of these two elements, oxygen and nitrogen, are capable of coördinating with water molecules by means of hydrogen bridges, since both have residual negative charges similar to those shown for the water molecule (23, 40, 41, 42). This type of tetrahedral coördination is demonstrated

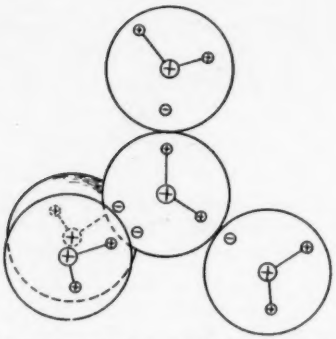


Fig. 12. Diagram showing four water molecules coördinated through hydrogen bridges to a fifth (21).

in Figure 12 where four coördinated water molecules are bridged to the fifth molecule in the center.

Apparently only a definite number may be coördinated with a particular oxygen or nitrogen group. Table VI shows the experimental and theoretical number for the most commonly found groups.

The total number which may be bridged to a chain of known composition may therefore be computed. This has been done by various investigators (24). We became interested in experimentally testing this computation; and in gelatin, where probably most of the chains are in the extended state, we found reasonable agreement in three or four different experimental procedures (25). The number of coördinated water molecules computed from the amino acid analysis checked with the amount of bound water; the x-ray evidence showed where the

TABLE VI

COÖRDINATION OF WATER MOLECULES WITH VARIOUS POLAR GROUPS

Polar Group	Number of Waters Coördinated		Reference
	Theoretical	Experimental	
H_2O	4	4	21
—OH	3	3	22
=O	2	2	22
$-C\begin{matrix}=O\\ \diagdown OH\end{matrix}$	4 or 5	4	23
NH_2	4	—	—
$-NH_2$	3	3	22
=NH	2	—	—
$\diagdown N \diagup$	1	—	—

bound water was located on the chain, and infra-red absorption studies (26) showed that it was attached through hydrogen bridges.

From evidence of this sort, where theory and experiment are in close agreement, we have gained confidence to carry our molecular picture into further details on higher dimensional levels. If now we wish to deal with a large protein particle consisting of chain lengths made up into a cube or into some other globular form, we have literally to step out of the molecular region, up into the lower range of the colloidal. A hydrated chain of 1000Å in length would, if folded, give rise to a globular particle about 50Å in size. It is true, we can measure this size and perhaps also measure somewhat larger particles by x-ray methods, but we are not very sure just what it is we are measuring, for there is very little clear cut confirmatory evidence to apply to the deductions; and the positions of the amino acid residues within these particles must be, to a very great extent, mostly conjecture.

But despite these apparent discouragements we may go considerably further with our picture, providing we recognize the tentative nature of our interpretations. Certain significant features of the *proteins* have come out of the great mass of research work which has been devoted to them; features which, it seems, can be reasonably applied to the *protoplasmic* picture. It must be recognized, and I should like to accent this point, that there is a great deal of difference between the deductions from investigations into the nature of *proteins* and deductions from researches into the nature of *protoplasm*. The former are essentially chemical; the latter are from studies of vital material. Both types are highly important, but must be viewed from strictly different angles. This comment is brought out by our next step in which we attempt to bring together the molecular and colloidal aspects as they may occur on these two levels in a minute bit of cytoplasm. Here again we wish to make use of a tentative conception in a simplified form, which, nevertheless, may be applied farther on to our later pictures of protoplasm.

When the colloidal aspects are introduced, three types of information are to be reconciled. The multiple question comes up—may we superimpose our molecular protein chain upon the protein particles obtained by ultra centrifuge, diffusion and other methods, and in turn again superimpose these upon the particles demonstrated by the ultramicroscope as existing in the protoplasm? If so, then we may feel that a starting point towards an understanding of a molecular structure in the protoplasm is at hand. Of these three phases of investigation the first, the molecular angle has been very briefly discussed, the other two are now to be considered.

In almost any microscopic view of active protoplasm minute granules may be seen suspended in a clear hyaline fluid, and as the magnifica-

tion is increased more minute particles come into view. A clear fluid free from particles is still apparent under the highest resolution in ordinary light, but when the shorter wave lengths of ultraviolet light are employed more particles are brought out (*27*) which were invisable in the white light. It seems probable that still smaller particles are present which could be made visible if means were available; that is, if still shorter wave lengths could be adapted. It is true, that by means of the ultramicroscope tiny flashes of ligh show the presence of hosts of minute particles whose existence is not shown by other means (*28, 29, 30, 31, 32*). In ordinary light the smallest resolved particle is about 5000Å while in ultraviolet light particles of about 2500Å may be demonstrated (*27*); but for determination of size below this we have at present no very satisfactory experimental methods, although various attempts have been made to determine the sizes of the particles which produce the flashes in the ultramicroscope. From these it is thought that the smallest particles of organic nature which would diffract light sufficiently to become detectable may be about 100Å in size (*29*). These particles have been shown to give protein reactions, and in the dried state to give, with x-rays, the characteristic 4.5Å and 10Å spacings (*16*). There seems to be no reason for a 100Å minimum limit here any more than for a 5000Å limit as shown by ordinary light, or 2500Å in ultraviolet light; and the electron microscope is offering considerable promise for smaller sizes. In fact it seems more rational to think that many particle sizes exist from the nucleus of the cell, down to molecular dimensions; and that the numbers increase enormously as the size becomes smaller. The size is limited in the lowest ranges by the configuration of the molecule and by the number of atoms it contains. The picture of cytoplasm then, for the moment, is that of particles of many sizes suspended in water. This becomes a little clearer when dimensions and numbers are employed.

Here we resort to two or three different ways of aiding visualization. We think of a 10 micron cube of cytoplasm; about 90% water and 10% dry matter, mostly proteins. We drain off the water and have left a small chunk of dry matter. By making use of the specific gravity of these two substances, water and dry matter, the volume of each is computed. We imagine the minute chunk of dry matter to be cut up into uniform pieces of ½ micron cubes, about 5000Å in size. The number of these minute cubes is, of course, determined by the size of the chunk obtained from the original 10 micron bit of cytoplasm. We now rebuild the 10 micron cube, diagrammatically, by placing these ½ micron cubes uniformly within the 10 micron cube. The space between the small cubes must account for the 90% water. Our interest here is centered on the spaces between particles. When all of the particles are assumed to be 5000Å cubes the spaces between them are found to be 3330Å. In

other words the depth of the channels between the particles is slightly less than the diameter of the particles. For pieces of 500Å, the channels are 330Å; and for 50Å particles, 33Å. In each case the proportions of the whole 10 micron cube remain 90% water and 10% dry matter; thus each particle may be considered as being accompanied by a shell of water which is proportional to its size.

This furnishes some rather interesting figures to which we shall return in a few moments. It is noteworthy, however, that particles of these sizes are known experimentally and have been shown to consist mostly of proteinaceous materials (33, 34, 35). Examples may be cited such as the viruses for the larger of the submicroscopic sizes, and particles in healthy cells for the smaller (27). The ultricentrifuge has shown that proteins may occur *in vitro* as particles of many sizes, and shapes of which globular particles of about 36,000 molecular weight are not uncommon (36). Thus our assumption of particle sizes has experimental background from, although not in, active protoplasm.

The protein particle of 36,000 molecular weight may correspond to one consisting of 1000Å of chain length. A thousand Ångstroms of chain, if hydrated, may be folded into a particle which approximates a 50Å cube, such as we imagined for the smaller particles of our 10 micron cube. If we now surround this 50Å cube with the 20,000 water molecules previously shown to be alloted to it in a 90% mixture, we shall find 6 layers of water molecules around each cube or 12 layers between cubes. The channels thus become 12 times 3, or 36Å deep, by

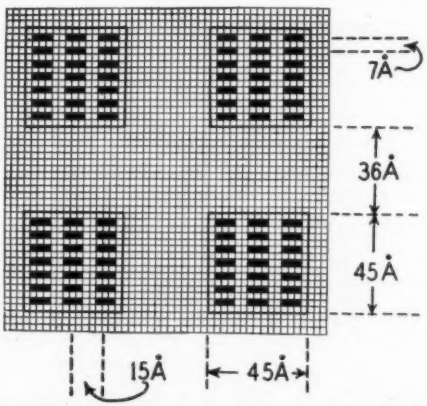


Fig. 13. Diagram showing four cubical protein particles separated by the proportional amount of water in a 90% water-10% protein mixture. Each particle is represented as consisting of about twenty short polypeptide chains, 50Å in length, of which only the end views are shown as solid black rectangles. The bound water is indicated by the small squares between the chains, and the free water by the greater number of similar squares between the large particles.

computation. This checks well enough with the 33Å just determined for the 10 micron cube of cytoplasm. The diagram in Figure 13 shows the proportion of water molecules, the small squares, to the protein chains which are shown in end view only as the solid rectangles. In the figure the larger groups of black rectangles represent 50Å particles in which the end views only of the folded short chain lengths are shown.

A somewhat clearer realization of molecular dimensions in a bit of cytoplasm and of the proportions of water to protein may be obtained by enlarging the 10 micron cube to a scale on which 1 micron equals 10 inches. This enlarges the 10 micron cube to the size of a small room 9 feet on a side. The 5000Å particles which are scarcely visible in the microscope are now enlarged to about the size of 5 inch grapefruits. These are placed uniformly about 3½ inches apart, and the spaces filled with particles of a fine powder, such as starch grains, to represent correspondingly enlarged water molecules. To simulate a mixture in which the smaller particles of 50Å occur, something about the size of a radish seed is made use of to replace the large grapefruits. The radish seeds are spaced uniformly at a distance not quite equal to their own diameter and again the spaces are filled with starch grains. One cubic foot of this radish seed starch construction may be considered as somewhat representative of one cubic micron under the microscope, where the radish seeds, reduced in size correspondly, would represent 50Å particles. About a hundred of these particles would form a linear row along the edge of the cubic micron, while nearly a million would be included in the cube. Each 50Å particle consists of protein chains equivalent to a length of about fifteen times that shown in Figure 6. With a million such protein molecular chains in a single cubic micron the amount of molecular material and the motion of molecular mechanisms becomes more easily realized. This cubic micron with its million protein molecules and many thousand times that many water molecules, it must be recalled, is the hyaline portion of the protoplasm, the transparent fluid free of visible particles.

When we consider this cubic micron of cytoplasm on a molecular scale with its 90% water and its million molecules of protein, we think of the water as partly within the particles, perhaps included in somewhat the same manner as indicated in gelatin, in the diagrams of Figure 14, where the groups of small circles represent end views of protein chains, and the larger circles between the groups are water molecules. In diagram C only about 40% water is present. This amount gives the particle a specific gravity of about 1.10 to 1.20, and is comparable to that of particles in active protoplasm (37, 16, 38 and 39). The mass as a whole in the cubic micron, just discussed, would have a density of 1.03 which is approximately the specific gravity of active protoplasm.

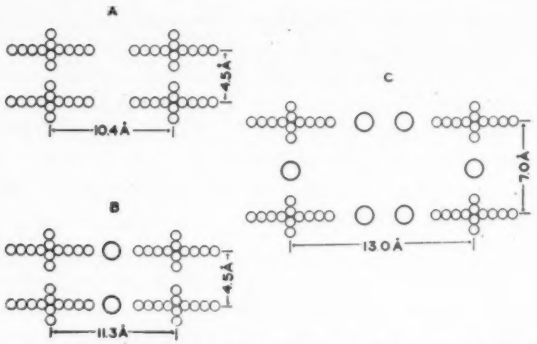


Fig. 14 Diagrams to show the hydration of gelatin (26). The groups of small circles represent end views of protein chains; the large circles between these groups correspond to water molecules. (A) Diagram of dehydrated protein packet; (B) Diagram of a packet containing about 15% water showing the hydration of the side-chain endings; (C) Diagram of a packet containing about 40% water showing the hydration of the backbones as well as the side chains.

The water channels between the submicroscopic particles will vary in width depending upon the density of a particular region within this cubic micron, since some of the particles may move close to one another. Where the channels are very narrow, it seems probable that the particles must come so closely in contact that they form compact aggregates. These we may assume to be approximately as dense as their component smaller particles; and may be large enough to become visible; that is, the aggregates may consist of a million protein molecules and thus become visible granules. But there is also the probability that the smaller submicroscopic particles may form less compact aggregates, or sponge-like clusters, having a density much lower than 1.10; these would have relatively wide internal channels of varying size and shape. With this low density they may be invisible, even though of large size, and be nearly comparable to a minute piece of a rather loose gel. Of the two types of particle aggregates, one a compact aggregate and the other a loose or spongy aggregation of smaller particles, there may be many sizes and shapes even within our single cubic micron of hyaline cytoplasm. That these exist seems to be a necessary consequence of the presence of the minute protein particles of 36,000 mol. wgt. or of smaller or larger, but still submicroscopic, particles of protein.

The picture, however, is not quite so simple as this would indicate, for in the channels, that is, in the water between particles, and within the sponge-like particles there occur ions and small molecules of organic substances. In our cubic micron the hydrogen ion concentration is likely to be about pH 6 to 7 (44, 45, 46); that is, the cubic micron will contain about 600 down to 60 H_3O^+ ions. At this concentration we

may expect to find the polar endings of the amino acid residues practically all ionized, with perhaps 20 to 40 plus and minus charges, respectively, on each protein particle of mol. wgt. 36,000. As a result of this we may expect to find certain specific local attractions and repulsions due to these charges; and also to find certain attractions where O and N atoms occur in the un-ionized state and form hydrogen bridges between particles. Both, the ionized and the un-ionized, become effective in holding the particles together.

Some conception of the strength of attachment between particles may be obtained by noting the number of places on the 50Å particle through which attachment may be made. Of the 300 residues there are only about 50 to 75 which have ionizable end groups and not quite all of these are ionized at pH 6 to 7. Lysine and arginine are almost certainly ionized; that is, have an extra proton or plus charge; histidine less certainly ionized. Glutamic and aspartic acids are also likely to be ionized; that is have a minus charge instead of the hydrogen proton on the carboxyl group. On a chain of 36,000 molecular weight there may be 20 to 40 plus and minus charges, respectively, as mentioned a moment ago, but when a chain is folded into a cube of 50Å, only half-a-dozen or so may occur on an outer face. Thus a face to face attachment between two particles could conceivably furnish only six or seven ionic bondings and then only if they were distributed in some exact manner. These particles, it must be recalled, are of 36,000 mol. wgt. and consist of about 5000 atoms, and may have only 6 ionic bonds to hold them together! The faces are 50Å square, and one must assume, in this case, that each plus group on one face occurs exactly opposite a minus group on the opposing face. The internal charges in the particle exert relatively little influence on the points of attraction, the charges, which are localized on the face.

Attraction of particle to particle is only one interesting effect of the presence of ions in the channels. Each ion, especially the ions, K^+, Na^+, Ca^+, etc., exert an individual influence in bringing the particles together as well as in keeping them apart. The charge on these monatomic ions is effective in attracting and orienting the water molecules into a shell surrounding each; and this water-ion aggregate may conceivably help to clog up the narrower channels and block off activities within a channel.

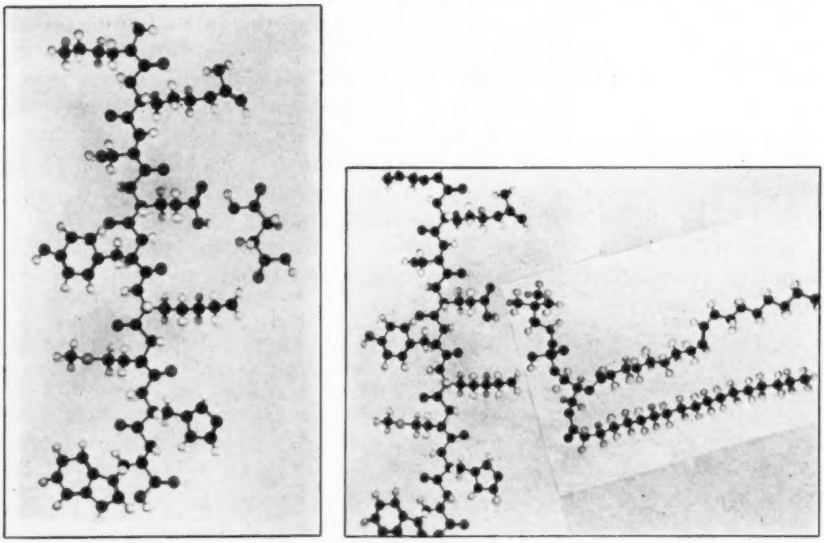
Further aggregation may take place in which the compact aggregates become larger and of various shapes; also in which the spongy aggregates may become larger and form appreciable gels; and still further, both sorts may be combined.

In these various combinations it seems reasonable that we shall find the vital mechanisms. For example, a chain of 36,000 mol. wgt. folded

into a cube will have a few ionizable groups on two or three faces. These may be distributed over the 50Å square surface in some fashion in which only one pair, of the six or seven present, is so located that it can become a place of attachment for a prosthetic group, thus converting the 50Å particle into an enzyme, let us say a specific respiratory protein. The chances are that there are not many places on a single protein of this size for such attachments.

One curious coincidence in connection with prosthetic groups and proteins is in the similarity of distances between the active attachment points. The distance between residues on one side of a protein chain is 7Å. It is the same between hydrated, adjacent chains. The distance between the known or most obvious points of attachment of a number of prosthetic groups is also very close to 7Å. This correspondence is shown in Figure 15. Such coincidences in structural dimensions leads one into speculation along several lines, always with the hope that such controlled imagination will point towards experimental methods for verification.

If we think of a small prosthetic group attached to a large protein molecule as being an enzyme and that several are necessary for completion of the respiration process, we may then think of a group of these



A B

Fig.15. A portion of a protein chain model is shown on the left in each figure. In A, succinic acid, the small molecule on the right, is the attached prosthetic group. In B, the active regions of lecithin are shown adjacent to two amino acid residue endings.

primary protein particles, each with its own prosthetic substituent, as forming one large spongy aggregate, a single gel particle but still far below visibility. This may have all of the necessary mechanisms and the proper organization of these mechanisms (43) for respiratory activity. There seems to be a certain necessity for the existence of such aggregates since some of the energy released by respiration must be used in the performance of work which is essential to the life of the cell.

Within our cubic micron, with its million protein molecules, we find ample space for many complete sets of respiration structures as well as channels leading to and from them which may aid in directing materials for the reactions. But we know very little yet of these submicroscopic granules, of the parts played by their components and of the localization of activities within them. A glimpse comes now and then from such activities as those which occur within a green plastid, a chloroplast in the green plants, where one set of reactions involves the chlorophyll and the protein components in synthesizing glucose molecules, while another set in the same particle of only a few microns in size is active in synthesizing a starch granule.

Obviously the static viewpoint must give way to the dynamic, but a dynamic which must include structures and guided reactions and activities. Up to the present we have had only an imperfect and static view of an attempt to visualize cytoplasm on a molecular and sub-microscopic level. Reconnaissance work along this line is still to be done on membrane structure in the cell and on the nucleus and its chromosomal components. Later the dynamic viewpoint must take precedence, for the vital processes must be activities which are resultants of a series of simpler localized reactions involving transformations of electron-proton systems. These systems we recognize as atoms, molecules and aggregates, which through their transformations become plastids, chromosomes and nuclei; whole cells, tissues and organisms complete. All, however, are molecular structures, or in the ultimate analysis, electron-proton systems.

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DISCUSSION REMARKS BY DOROTHY WRINCH

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A number of points raised by Professor Sponsler in his paper received attention in the discussion. The consensus of opinion was that the nature of the protein is the central theme in the problem of protoplasmic structure and that the further development of this special and fundamental aspect of physiological chemistry, by means of all the available techniques, including those of biology, immuno- and enzyme and virus-chemistry, of x-ray and electron-diffraction analysis, offers the best, perhaps the only way of bridging the gap between the angstromworld of atoms and the world of microns.

The essential difficulty of contenting ourselves with the traditional picture of proteins as long chain structures, in view of the fact that the vast accumulations of evidence regarding the protein discover an essential difference between certain biologically active proteins and the long chain structures familiar in (e. g.) cellulose studies, was pointed out by more than one speaker. While there may still be some gaps in the final proof that such proteins are definite chemical entities, there seems no doubt that they are characterized by a high degree of organization. The importance of attempting to build into one consistent picture what this concept connotes in biology on the one hand and in chemistry, physics and mathematics on the other, was stressed. So far as the molecular aspect is concerned, it seems that a high degee of organization is a symptom of a special type of atomic arrangement. The existence of megamolecular structures with personalities as definite as those of the ordinary molecules of organic chemistry provides a new problem for chemistry and physics as well as for biology and it was shown that the idea that the essential entity in proteins is an atomic fabric (as in the cyclol theory) offers a solution of the problem. Fibrous protein structures which play so important a role in protoplasm were discussed. These, I would suggest, may also be built on a fabric motif. Thus characteristic protein fabrics, having an atomic pattern not at present directly deducible from experiment, provide a unified approach to proteins whether fibrous or globular. The latter, which appear to be responsible for the specificities of protolasm, may owe their chemical individuality to closed surface structures in which certain numbers of individual amino acid building blocks and those numbers only can be accomodated, thereby explaining the molecular weight classes of the globular proteins and their high degree of organization. (The number 288 used by Professor Sponsler in some of his analyses derived by closed cyclol structures is an example).

The great importance of the part played by water molecules in determining orientations in protoplasmic structures was discussed by several speakers with special reference to the striking figures given by Professor Sponsler for the proportion of water to protein in protoplasm.

THE SYNTHESIS OF PROTOPLASM CONSTITUENTS*

(An Abstract)

By

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The terms growth and biological synthesis designate similar processes, namely the new appearance of morphological or chemical units. Both are the result of the interaction of exogenous material (food) with the specific organic cell constituents, most of which represent large and complex molecules (proteins, lipoids and polysaccharides, etc.). In the absence of growth, when body weight is unaltered, the total amounts and distribution of these components remains constant within narrow limits. The prevalent view that these substances are mainly stationary and stay unaltered has recently been challenged. The application of isotopic compounds has shown that all constituents of the living cell, even those which are regarded as structural elements, are involved in continuous and rapid chemical reactions which result in a balanced synthesis and destruction. New formation of body material, i. e. synthesis, is thus not restricted to the condition of growth, but proceeds rapidly at all times.

The only tools for the investigation of chemical reactions occurring in systems that do not change total amounts or composition, are isotopes. The rapid chemical reactions in which proteins and fats are involved have been disclosed by adding to the normal stock diet of adult rats physiological fatty acids or amino acids, etc. which had been synthesized in the laboratory in such a way that one or more of their constituent atoms were largely present in the form of their respective heavy isotope: Hydrogen atoms were rich in deuterium and nitrogen in N^{15}. Such compounds are practically indistinguishable from their natural analogues and the animal and plant cells treat both indiscriminately. The chemist, however, by atomic weight determination, can not only follow the pathway taken in the animal organism by a given food constituent, but in many cases can establish the chemical reactions in which it was involved and also determine the other molecules with which it had interacted. If an isotopic compound A has been administered to an animal or a plant, and an isotopic compound B is in turn

*Most of the work on which the paper is based has been carried out in collaboration with D. Rittenberg, S. Ratner, K. Bloch, A. S. Keston, D. Stetten, R. Moss, M. Roloff and F. W. Barnes. A review of the experimental findings has appeared in Physiological Review, **20**: 218, 1940.

found in the organism, the isotope content in B is proof that A was either converted into B or had interacted with B in a way which involves a transfer of an atom or of a molecular grouping from A to B.

When a small amount of an isotopic amino acid containing N^{15} in the amino group is given as a component of a normal diet to an adult, non-growing rat, the nitrogen of the proteins of the animals is found to contain a large part (40 to 60 per cent) of the isotope while only moderate amounts of the isotopic nitrogen are recovered in the nitrogen of the small molecules (non-protein nitrogen) or in the excreta. All physiological amino acids investigated so far (glycine, tyrosine, leucine, glutamic acid, and arginine) showed this effect, which must therefore be typical of all dietary amino acids. The result shows that despite the unchanged total amounts and composition of the animals' proteins they must have been involved in rapid and extensive chemical reactions.

Isotope determination of the split products (amino acids) of the proteins from various organs of the animals has revealed the type of reactions to which the proteins were subjected.

(1) Part of the dietary amino acid had entered the protein molecule replacing the same type of amino acid in the proteins (leucine had replaced leucine, etc.).

(2) α-Nitrogen of one type of amino acid of the protein had been removed by deamination and had in turn been transferred by amination to other types which had lost their nitrogen. This process results in a constant wandering of the α-nitrogen from one amino acid to others in the proteins.

(3) Part of the nitrogen of the α-amino acids had been transferred to the amidine group of the arginine of the proteins, this amidine group is continuously replaced and the removed amidine group appears in the urine as urea. The amidine group of the arginine of the protein molecule is thus one of the stations in the "overflow" of nitrogen in mammals.

(4) The carbon chains of many amino acids of the proteins are constantly converted into those of others, i. e. phenylalanine is converted into tyrosine, ornithine into proline and glutamic acid, etc. The newly formed free amino acid, like those from the diet then replace the same type of unit in the protein molecule.

All these reactions of the cell proteins are automatic and are not suppressed by dietary changes, e. g. some phenylalanine is always converted into tyrosine even if the animal is overfed with tyrosine. The newly formed amino acids are then introduced into those sites of the protein molecule which are always occupied by tyrosine.

The occurrence of these complex reactions forces the conclusion that in the living cell the peptide linkages of the proteins which unite the amino acids are constantly opened, thereby liberating amino acids. The latter merge with the analogous free amino acids originating from cell proteins or diet, and interact chemically with other types of amino acids. The new reaction products reenter into vacant spaces when peptide linkages are again closed. These processes are so balanced that they do not lead to an ultimate change of the system, i. e. of the total amount or composition of the cell.

While the various proteins react at different rates, it is questionable whether there exists in the living cell any protein that stays entirely outside this continuous chemical interaction of food and body constituents. As has been shown in collaboration with Professor M. Heidelberger, (unpublished) even such a highly specific protein as the pneumococcus antibody (Type III) in rabbit serum is involved in the same continuous chemical reactions as the proteins of internal organs and muscle.

Not only the amino acids but also other protein constituents display such extensive chemical activities. The nucleoproteins, which are known to be specific components of nuclear structures, contain purines. Birds, which in contrast to mammals excrete nitrogen mainly in the form of the purine derivative uric acid, show an extremely rapid replacement of protein purines by newly formed purines. If they are given small amounts of isotopic ammonia, the purines of the nucleoproteins acquire a high isotope content. The finding can scarcely be interpreted in any other way than that purines are continuously formed from ammonia. These newly formed compounds replace the purines bound to nucleoproteins, and the liberated purines are converted into uric acid, which is excreted. The purines of the nucleoproteins have thus to be regarded as intermediates in the formation of uric acid.

This reactivity is not restricted to animal proteins but is also observed in plants. Preliminary experiments carried out with Dr. Vickery of the Connecticut Agricultural Experiment Station in which tobacco plants were kept on a nutrient containing isotopic ammonia demonstrated a similar reactivity of the plant proteins. The specific reactions observed were almost the same as those in animals.

Analogous experiments in which isotopic fatty acids were given to animals showed that the fats of the animals are also involved in a multitude of rapid automatic chemical processes. The ester linkage uniting glycerol and fatty acids is continuously opened and the fatty acids likewise mix with the same type of dietary fatty acids. Part of this mixture may enter again into ester linkage, while another part of the mixture is subjected to chemical reactions. Some acids are dehydrogenated to

form unsaturated acids, while simultaneously an equivalent amount of acid is hydrogenated. The chain of other acids is degraded by two or four carbon atoms with the concurrent elongation of other chains by the same number of carbon atoms. The newly formed compounds also enter ester linkages to become constituents of body fats. Like the reactions of the proteins and amino acids, those rapid reactions of the fats are also balanced in such a way as to avoid change in total amount and composition.

The application of isotopic molecules has shown that all, or almost all, chemical reactions, synthetic or degradative, which the cell is able to carry out, proceed all the time at rapid rates. If food enters the organism its split products merge immediately with the identical compounds which are liberated automatically from the large molecules of the cell constituents. The material that is ultimately metabolized and recovered as carbon dioxide, urea, etc., does not represent merely food material, but is a sample of the metabolic mixture originating from the chemical interaction of dietary material with the large amounts of reactive cell components.

The constancy of cell structure is thus only apparent, as the component elements are in automatic chemical interaction and are replaced rapidly. The continuous liberation of small units from larger molecules renders their "active" groupings available for metabolic cycles. The large molecules of the protoplasm thus have a double function, structural and metabolic.

DISCUSSION REMARKS

To Rudolf Schoenheimer's Paper On "Synthesis Of Protoplasm Constituents"

By

Otto Glaser

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The use of isotopes to detect chemical transformations or the reciprocal exchange of specific groups or particular atoms from one molecule to another, calls for methods and devices both difficult, and novel. To discuss these intelligently, requires very special skill. One technical detail however cannot be omitted. The tracers were given to normal rats maintained on normal diets. In these only a fraction of one of the dietary constituents was modified. Thus the total isotope dosage administered per individual was never allowed to exceed an

amount to which the animals are entirely indifferent. Mainly for this reason, Schoenheimer's results project from their immediate field of origin into the realm of genetics, including development and growth and impinge on our general ideas about organization. Here "tracer-metabolism" becomes a vigorous semantic purge.

For the student of growth, proof that the stationary state of the adult involves a metabolism characteristic also of embryos, is a true emancipation. Rapid and wide-spread syntheses occur as long as the organism lives. The increments of youthful growth are the outcome of imbalance between synthesis and destruction. This imbalance is self-corrective. Instead of postulating "high growth potentials" we must hunt for mechanisms that control shifts in equilibrium. Are we to look for additions, subtractions, or modifications of enzymes, or of substrates, or of both?

Not only are individual atoms or small groups constantly vacillating between any two amino-acids but entire peptide links may be replaced by free units of the same type. These units split off either from proteins resident in the organism or from immigrant dietary molecules. In birds isotopic ammonia registers in the purines present in nucleoproteins. Moreover purines when free replace their like in nucleoproteins. Hence the latter become intermediates in the formation of uric acid. These facts with their genetic bearing point with equal directness toward the spatial and dynamic configurations of the molecules themselves. At this level where we must reckon with the constant opening and closing of peptide linkages there is some chance that certain statements about "native" and "denatured" proteins may prove identical with analogous assertions about the "living" proteins and the "dead".

Equally frequent exchanges, replacements, degradations and syntheses are demonstrable on fatty acids and fats. But carbohydrates transform into fats and deaminized protein residues into carbohydrates. Thus within the living cell there are no barriers to free-trade. All possible reactions occur among the inorganic constituents; between these and the organic; and among the organic themselves. In time then, a change in any molecule may reverberate throughout the entire system and affect some other molecule, spatially and taxonomically remote. This general vision is neither accidental nor unique. In the long run it develops in all fields of biology. Compartment psychology and absolute cerebral localization are now things of the past; the detached reflex is recognized to have little in common with the same reflex *in situ* while ductless glands once condemned to work apart have been assigned their proper rôles in an endocrine system. On the ninth day of incubation to the accompaniment of a new method of respiration the growing chick increases its rates of calcium accession; of synthesis, deposition and

destruction of proteins and fats; and flattens out the curve of cumulative acceleration in heart-frequency. Moreover the temporal interrelations among these events, their immediate respective precursors and consequences remain obscure unless all, singly and in association, be correctly timed.

The enormous success of modern genetics is perhaps the most brilliant example of the same principle. In this case there are no results unless the organism remains entire. Chromosomal alterations and behavior are not often recorded until we have the adults of the F_1 or F_2 generations. In practice, in either case, mutations reassortments, inversions, crossovers and the like register only in forms and places far removed from their genic level of origin. Through numerous refinements and complications these facts have led to the discovery of complementary genes, of position-effects and the downfall of the absolute sovereignty once attributed to the individual gene. The dictators of development and regeneration should take notice. How long before we shall strip the verbal pomp off evocators, inductors, organizers and "fields" and force the naked residues to accept stations appropriate in a molecular democracy?

The organism as a whole is not easy to understand, but it happens to be the only organism there is. Why then are we so frequently surprised when it asserts itself? The answer is imbedded in our methodology. With the data available at the time probably no one could have justified the insertion of Vitamin A into the visual process. Ignorance then is one thing. But too many of our surprises come about because we first build up elaborate systems of mistakes. Most of these trace to isolation experiments in which the whole is subsequently forgotten. Against this fallacy of the undistributed middle the biologist has two modes of insurance; in the one he is forced to think his detachable fraction back into the situation from which it came—a technique that presupposes knowledge of the situation; or simpler still, he keeps his organism continuously intact. Unless these things are done, sooner or later the organism revenges itself by becoming utterly bewildering. In this predicament the old metaphysical cures are now impotent. Shall we bay at or deny the organismic moon? It is most unlikely that an organism could be organized at one level without being organized at all levels. By facilitating the extension of this concept into the molecular and atomic realms Schoenheimer leaves no room for a specifically biological mysticism. Instead he enables us to attach very precise objective meanings to the assertion that an organism in its spatial and temporal aspects is always a system devoid of isolated events. This system constitutes the one problem which no biologist can escape.

COLLOID CHEMISTRY OF DEVELOPMENT AND GROWTH

By

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INTRODUCTION

Before discussing the subject of my talk, I would like to make some remarks about the structural units of organisms and about some points of colloidal optics. The structural units I mean are not those of the biologist, but those interesting the physico-chemist. We presumably never find that simple and uniform structure as in pure liquids or crystals, where over a macroscopic or, at least, microscopic range the same structural units, atoms, ions or molecules, repeat themselves over and over again. In organisms we are practically always dealing with a much more involved structure. Let me start, rather arbitrarily, with a table given by Frey-Wyssling (1) (Table I) containing the structural elements of the cell membrane of cotton: there are the amicronic elements, like the C-atom and other atoms, and molecular groups of atoms, like the residues of glucose and of cellobiose, as we have them also in other organic compounds. These small elements unite to the first of the much larger elements, namely to the long primary valence

TABLE I

DIMENSIONS OF STRUCTURAL ELEMENTS IN THE CELL WALL OF COTTON

Order of magnitude as to length	Structural element	Breadth	Length	Remarks
amicronic 1-100 Å	C-atom	1.55 Å	1.55 Å	
	glucose residue	1.5 Å	5.2 Å	
	cellobiose residue	1.5 Å	10.3 Å	
submicronic 100-1000 Å	primary valence chain	1.5 Å	undefined, >1000 Å	1. growth in length of primary valence chains.
	micelle	50 Å	500-100 Å	2. oriented clustering of primary valence chains and of micelles.
microscopic 0.0-100 μ	fibril	0.4 μ	undefined	
	lamella	0.4 μ	undefined	
macroscopic >0.1 mm.	layer	0.01 mm.	50 mm.	3. growth of tissues and in tissues.
	fibre	0.01 mm.	50 mm.	

*Professor Freundlich died before his manuscript was set up in type; the printing has been supervised by his colleagues.

chains, like those of cellulose, starch, chitin, rubber, the proteins and many other substances, so characteristic of organisms; their breadth is that of atoms or molecules, their length very much greater, reaching far into the range of colloidal size. In many cases long primary valence chains vary strongly as to length and, hence, as to size; when they are formed in equal size, like in proteins, we speak of macromolecules. Beginning with this structural element, we are dealing in the whole rest of Table I with colloidal systems, the long primary valence chains or, in other cases, the micelles, i. e. micro- or quasi-crystalline groupings of these chains, being the units of the larger structural elements. From a colloidal point of view these larger elements, fibrils etc., are gels, generally, it is true, very complicated gels, but combining, like them, the properties of the liquid and the solid state and showing phenomena such as swelling etc.

It is generally acknowledged that long primary valence chains exist in the substances building up the framework of organisms, i. e. in structures like the cell membranes of plants or the chitin coatings of insects, and also in stored substances such as starch grains or rubber particles. But, in my opinion, there is no doubt that those are right who assume that long primary valence chains also exist in protoplasm (2), i. e. in all those parts of organisms, where the processes determining life are actually going on. Two facts prove this assertion: protoplasm shows structural viscosity (3), i. e. its viscous behavior does not obey Poiseuille's law, or more correctly, Newton's law and it may be changed by mechanical means such as shaking or stirring. Low values of viscosity, i. e. the true viscosity is found only if the protoplasm is exposed to high rates of shear. Structural viscosity becomes also conspicuous by the fact that the apparently high viscosity of protoplasm does not cause a corresponding decrease of its electrical conductivity. According to Gelfan (4) the conductivity of protoplasm would correspond to that of a 0.05n KCl solution, if we assume its true viscosity to be in the order of that of water; if the apparent higher viscosity were true viscosity—as it would be if caused by a substance such as glycerol—the observed electrical conductivity would correspond to that of a 500n KCl solution, a most improbable result. Sufficiently concentrated solutions of long primary valence chains practically always show structural viscosity.

Perhaps even more convincing is the second fact, namely that protoplasm itself may be double refracting. This was observed in strands of protoplasm in plant cells (5) and also in amoebae (6). Double refraction occurs in liquid or semi-liquid systems like protoplasm only if they contain long primary valence chains or micelles.—How far micelles are actually a constituent of cytoplasm is perhaps an open question. Some authors emphasize that cytoplasm is more or less

homogeneous under the ultramicroscope (*7*), a fact which would make the presence of micelles improbable, but which would not be inconsistent with the presence of long primary valence chains. Other authors do not hesitate to assume both long primary valence chains and micelles in the cytoplasm.

I have mentioned double refraction and I shall have to refer so often to this phenomenon in my talk that I would like to discuss briefly some important properties of double refraction and dichroism (*8*).

A beam of monochromatic light passing through a prism made from an amorphous substance like glass shows the well known path first investigated by Newton. If we replace the glass prism by one cut from a uniaxial crystal, e. g. calc-spar, and if it is cut so that the optical axis is perpendicular to the triangular cross section, two waves of light pass through the crystal which has different indices of refraction: the two beams are polarised at right angles to each other. The double refraction δ is simply the difference of the two indices of refraction n_e and n_o:

$$\delta = n_e - n_o$$

n_e belongs to the so-called extraordinary ray whose vibrations are parallel to the optical axis, n_o to the ordinary ray whose vibrations are perpendicular to the optical axis. Double refraction is called positive, if $n_e > n_o$, negative, if $n_e < n_o$.

It is a result of this behavior that a plate of a uniaxial crystal cut parallel to the optical axis does not remain dark between crossed nicols in all positions, but allows light to pass through, when the optical axis is not perpendicular or parallel to the plane of polarization.

The two beams passing through the crystalline prism may also be absorbed to a different degree, a phenomenon called dichroism. We have positive dichroism, if the extraordinary ray is more strongly absorbed than the ordinary ray, negative dichroism, when the opposite is the case.

Optical anisotropy includes both double refraction and dichroism.

We can understand readily the optical anisotropy of colloidal systems, i. e. of sols and gels, using a similar device. As a prism we have a glass tube of triangular cross section and we arrange the experiment so that a liquid in the tube may be at rest or flow. If the tube is filled with pure water or with any pure liquid or true solution, we observe an isotropic behavior, i. e. the prism behaves like one of glass, irrespective of whether the liquid is at rest or is flowing. But if the tube is filled with a suitable colloidal solution, for instance with an aqueous sol of V_2O_5 or of myosin, the behavior is very different: as long as the liquid is at

rest, it does not differ from water. But if it is allowed to flow, the beam of light is split into two beams polarised perpendicularly to each other; we observe stream double refraction. All that was said about the positive or negative sign and about dichroism also holds for the optical anisotropy of sols caused by streaming.

Stream double refraction is never observed if the particles of the sol are spherical; they must be non-spherical, rod-shaped or plate-like, to cause this phenomenon. I shall simplify matters by referring mainly to the case of rod-shaped particles. Stream double refraction is readily understood if we make the further simplifying assumption that the particles are very small, rod-shaped, uniaxial crystals with their optical axis parallel to their long axis. As long as the liquid is at rest, no double refraction is observed: the particles are distributed quite irregularly, moving about owing to their Brownian movement, and the optical effect caused by particles just lying in one direction is compensated by that of particles just lying in a direction perpendicular to the first. But if the sol is flowing, then, owing to the velocity gradient in the liquid, the particles are to a certain percentage or even completely oriented, generally with their long axes more or less parallel to the lines of flow.—The degree of orientation depends mainly on the size and intensity of the Brownian movement of the particles.—Hence, a tube, with a rectangular cross section, full of a streaming liquid containing very small, rod-shaped, uniaxial crystals with their long axes parallel to the lines of flow would behave like a plate cut from a uniaxial crystal parallel to its optical axis.

This is the one distinct case of stream double refraction, where it is due to the crystalline structure of the particles, i. e. it is due to their intrinsic double refraction. A second distinct case is no less important: if the colloidal particles are naturally not double refracting at all, being amorphous or belonging to the regular crystal system which does not show double refraction, their colloidal solution can, nevertheless, be stream double refracting, if the particles are non-spherical, regularly oriented, and small compared with the wave-length of light. If the liquid contains, for instance, very small rods of amorphous glass which, when the liquid is flowing, are oriented parallel to the lines of flow, it becomes double refracting, provided that the difference in the refractive indices of the particles and the liquid is large enough. This is the so-called morphic double refraction. In the case of rod-shaped particles the double refraction may be calculated according to an equation given by O. Wiener (9):

$$n_e^2 - n_o^2 = \frac{\zeta_1 \zeta_2 (n_1^2 - n_2^2)^2}{\zeta_1 n_2^2 + \zeta_2 n_1^2 + n_2^2}$$

Here n_e and n_o have a meaning analogous to the above. n_e is here the refractive index of the dispersed system parallel to the orienting force i. e. parallel to the optical axis of the dispersed system; n_o is the refractive index perpendicular to it. n_1 and n_2 are the refractive indices of the particles and the medium between them; ζ_1 and ζ_o are the relative volume concentrations of particles and medium. Obviously the morphic double refraction of the rod-shaped particles is always positive. The morphic double refraction of plate-like particles is always negative if the plates are arranged in planes parallel to each other.

Systems of oriented, small, non-spherical particles may also show morphic dichroism.

The optical anisotropy of colloidal solutions is very frequently made up of both intrinsic and morphic double refraction and dichroism. In some cases, for instance in colloidal solutions of tobacco mosaic virus, the double refraction is apparently only morphic (10): it disappears, in agreement with Wiener's equation just mentioned, if the refractive index of the liquid is made equal to that of the particles. Hence, the latter do not seem to have a truly crystalline structure.

As will be discussed more extensively later, double refraction may also occur spontaneously in sols containing non-spherical particles, provided that they are sufficiently concentrated. Then the particles may orient themselves parallel to each other without external incitement (11).

It is now readily understood that the double refraction of protoplasm definitely proves that it contains non-spherical particles, most probably long primary valence chains.

A few words must be added concerning the double refraction of gels. In sols, streaming is the main factor causing orientation of the particles and, hence, optical anisotropy; in gels the same end is achieved by stretching. In some, comparatively simple natural gels like cellulose fibres, very small crystalline—or quasi-crystalline—particles are oriented from the outset practically parallel to the long axis of the fibre. Hence, they are forthwith double refracting, showing both intrinsic and morphic double refraction. Unstretched rubber, on the other hand, is not double refracting but becomes so on being stretched (12). The behavior of rubber indicates that it contains primary valence chains of varying length coiled up and distributed quite irregularly. On stretching, the chains are first uncoiled, then extended and oriented, thus producing first double refracting groups of long chains, finally crystalline micellae (13). In gels it is easier to distinguish experimentally between intrinsic and morphic double refraction (14): the gel stretched (or unstretched) only need be soaked in liquids having different refractive indices. If double refraction disappears, when the

refractive index of the liquid is equal to that of the particles, double refraction is merely morphic. If a minimum value is reached with a certain refractive index of the liquid, then this value corresponds to the (positive or negative) intrinsic double refraction of the particles.

With gels, in some cases a third kind of double refraction has to be taken into account: one caused purely mechanically by stretching extended particles still farther, a kind of double refraction as it is observed, if glass is stretched or pressed. But, as a rule, intrinsic and morphic double refraction are responsible for by far the greater part of the double refraction observed.

In gels more readily than in sols, X-ray investigation may be used for confirming the results gained by investigating optical anisotropy and for extending them further into details of crystal structure and orientation.

Colloidal processes in development and growth.

Turning, at last, to the subject of my talk and to Table I, we may look for the most probable kinetic processes in the range of colloidal structures. We have

1. The growth of small molecules to long primary valence chains and the further growth of these chains in length;

2a. The clustering of primary valence chains; they may form micelles or other structures similar to those of liquid crystals;

2b. In the same way micelles may form clusters, when they are more or less parallel to each other.

If the conception of W. M. Stanley is correct that tobacco mosaic virus and other similar organisms may be considered to be macromolecules of nucleoproteids, then the reproduction of macromolecules would be presumably the most important process of development and growth occurring with structural elements of this size. It would have to be discussed here.

3 Processes concerned with gels: growth influenced by the orientation of particles in gels or by the way chemical reactions occur in them: processes influenced by osmotic effects or by swelling etc.

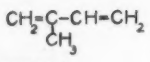
These points will be discussed singly; it is, of course, true that our knowledge is poor and fragmentary.

Growth of primary valence chains.

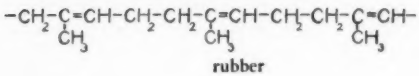
Chains may be produced by two types of reactions, by polymerisation and by condensation. In polymerisation small molecules are simply strung together. The formation of rubber from isoprene and of polystyrene from styrene are reactions of this kind (cf. Table II). In condensation the molecules forming the chain undergo dehydration or a similar kind of reaction. The formation of starch or cellulose from

TABLE II

Polymerisation

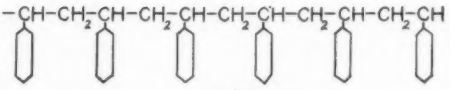


isoprene



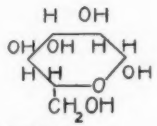
rubber

styrene

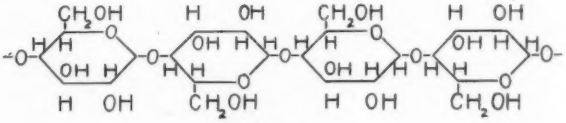


polystyrene

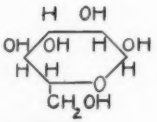
Condensation



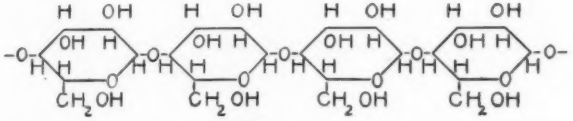
β-glucose



cellulose



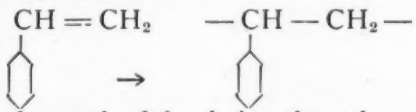
α-glucose



starch

α- or β-glucose respectively (cf. Table II) and that of proteins from amino acids belong to this group.

It is well known that the kinetics of chemical reactions is very specific and does not lend itself readily to generalizations. I, therefore, make the following remarks with due care, the more so as the experimental results and the conceptions of different authors on this field do not agree very well. So far, reactions of this kind have mainly been investigated in practically homogeneous solutions (15), e. g. the polymerisation of styrene by heat, where the unpolymerised (or weakly polymerised) molecules behave as the solvent. Apparently there are three fundamental processes going on: the initiating reaction, where the styrene is split open to a radical;



secondly, the actual growth of the chain, where the radical first formed

reacts with other molecules, the growing chain always keeping up its radical structure; finally, the reaction causing the chain to come to an end, a special end-group of the long chain being formed. With styrene, the initiating reaction appears to be the slowest, thus impressing its rate upon the whole process. The last reaction causing the chain to come to an end is still especially puzzling and important. In the case of polystyrenes a double bond seems to be formed at the end.

This third process, particularly, but also the other partial processes of these kinds of reactions are strongly sensitive both to positive and to inhibiting catalysts.

If we knew more about the reactions causing the chains to come to an end, we could answer some questions which are especially interesting, but which, as far as I can see, have not yet been answered satisfactorily: why do the chains come to an end at all? Why do we find in many cases (polystyrenes, rubber, cellulose etc.) primary valence chains varying greatly in length, whereas the polypeptide chains of the proteins so readily form macromolecules of a well defined molecular weight?

Now, this whole reasoning is marred by the fact that all results refer to reactions in practically homogeneous solutions, whereas it is nearly certain that reactions in organisms and, probably, the formation of long primary valence chains too, occur at interfaces, a point which was already emphasized by Carothers (16).

It is not very probable that the growth of long primary valence chains becomes conspicuous as a direct phenomenon of growth in an organism. According to some authors (e. g. van Iterson (17)) the apposition of cellulose in some plant cell membranes may be due to a direct growth of cellulose chains on the membrane.

Clustering of primary valence chains and of micellae.

The following processes are less chemical, but more colloidal or physico-chemical. Long primary valence chains lying parallel to each other may unite to small crystalline units, the micellae, as was mentioned above in the case of rubber. Both long primary valence chains and micellae may form larger clusters enclosing a certain amount of the liquid medium and of other substances leading to phenomena which in many respects may be important to the development of organisms. Although it is perhaps not quite consistent, the behavior of micelles will be discussed first, because it allows us to make use of some purely colloidal phenomena.

As was briefly mentioned above, the non-spherical particles of sols can be oriented not only by letting the liquid flow, but in sufficiently concentrated sols they may orient themselves spontaneously (11). This has been observed particularly in colloidal solutions of V_2O_5 and of dyestuffs like benzopurpurin etc. whose particles may be looked at as

micellae. A thin layer of a concentrated $V_2 O_5$ sol under the polarization microscope between crossed nicols is at first dark, because the rod-shaped particles are distributed quite irregularly. But in course of time flame- or spindle-shaped double refracting spots appear. If the liquid is stirred, they disappear, but they are formed again, when the liquid is left to itself. In these spots the rod-shaped particles are oriented practically parallel to each other, only converging at the two ends. This can be shown by comparing one and the same spot under the polarization microscope and under the ultramicroscope (cf. Fig. 1 and 2).—In Fig. 1, A and P indicate the direction of polarization of the analyzing and polarizing nicol. In Fig. 2, I indicates the direction of the illuminating light.—In the latter, the spots are only visible distinctly, if the illuminating light strikes the micelles perpendicularly to their long axis; in this way the position of the rods can be determined.

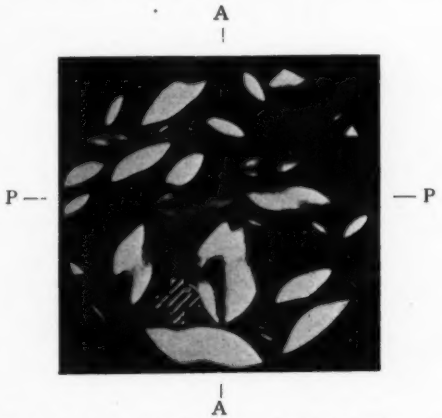


Fig. 1. Tactoids in a $V_2 O_5$ sol under the polarization microscope (H. Zocher).

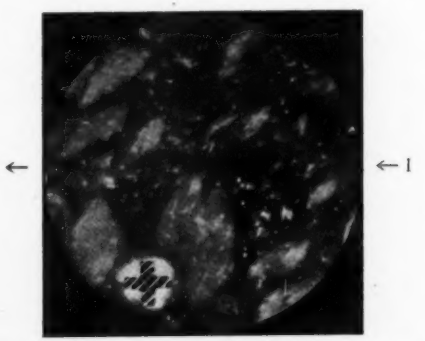


Fig. 2. The same tactoids as in Fig. 1 under the ultramicroscope (H. Zocher).

Fig. 3. Tactoids in suspensions of tobacco mosaic virus (F. C. Bawden and collaborators).

Very similar spots are observed in colloidal suspensions of tobacco mosaic virus whose particles are strongly rod-shaped forming suspensions very similar to V_2O_5 sols (18). Fig. 3 shows the spontaneous double refraction of virus particles under the polarization microscope.

These spots are frequently called liquid crystals. This term does not seem quite correct to me. The systems, generally called liquid crystals, are formed on fusing substances such as azoxy-phenetol or azoxy-anisol etc. Here we are dealing with pure substances having rod-shaped molecules which may be oriented parallel to each other, thus forming liquids which are double refracting and which have other crystalline properties. They differ in two points from the "liquid crystals" in colloidal solutions. In the true liquid crystals, only one kind of molecules is present, not so in the micellar liquid crystals, where the rod-shaped particles are separated from each other by thin layers of the medium. Secondly, the true liquid crystals represent phases which exist only in a certain range of temperatures, e. g. azoxy-phenetol between 135°-165° C. Nothing of this kind is observed in the micellar liquid crystals. I prefer to call them tactoids.

Their formation appears to be closely related to the phenomenon known as coacervation. In the limited sense of the word, as it was first defined by Kruyt and Bungenberg de Jong (19), coacervation refers to the formation of a new liquid phase, generally richer as to colloid, in a colloidal solution of one or more hydrophilic colloids. Coacervation occurs, for instance, in mixed solutions of gum arabic and gelatin of suitable concentration and pH or in solutions of gum arabic containing a certain amount of the so-called hexol cation, a hexavalent complex cobalt cation. The coacervation in solution of two oppositely charged colloids is known as complex coacervation, that in solution of one colloid and an ion of opposite charge is one type of autocomplex coacervation.

Now a tactoid behaves as a new phase. In sufficiently concentrated sols of V_2O_5 or of tobacco mosaic virus the tactoids formed may settle down and form a new phase which is separated by a distinct boundary from the upper phase and richer in colloid (20). The lower phase of tactoids—frequently called the tactosol—is spontaneously double refracting, whereas the upper phase only becomes double refracting on streaming. Langmuir (21) does not hesitate to call the formation of such an optically anisotropic, liquid crystalline phase, instead of a normally isotropic liquid phase, a coacervate. As we are dealing with only one kind of colloid, we would have to consider it an autocomplex coacervate.

There is still a great uncertainty as to the forces uniting colloidal particles in coacervates (22). The sensitivity towards electrolytes indicates that the tactoids of V_2O_5 depend in some way on the action of electrical forces working between the particles themselves and the ionic atmospheres between them. As was mentioned above, there is always a layer of the medium of a certain thickness between the particles. It is still a point of controversy, whether a high degree of hydration of the particles accounts for the thickness of these layers or if the electrical forces alone are responsible both for the attraction between the particles and for their remaining so far apart that thick films of medium remain in between.

There are phenomena in the development of organisms, where processes of this kind may be essential, though the units in question are probably not micelles, but primary valence chains.

Among the very many structures in organisms which are spontaneously double refracting, there is one which reminds one forthwith of tactoids and their formation. It is the spindle formed in mitosis (23). It is not only the similarity in shape, the spindle has been found to be double refracting in a number of cases. This was first observed by Runnström (24) and then by others, particularly by W. J. Schmidt (25). In Fig. 4 a photograph given by the latter of a segmenting egg

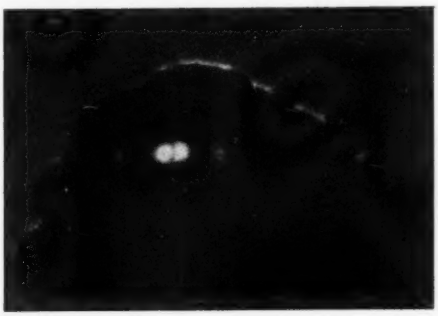


Fig. 4. Double refraction in mitosis (W. J. Schmidt).

of a sea-urchin (Psammechinus miliaris) is shown between crossed nicols. The strongly lighted up points are the double refracting chromosomes; the weakly lighted up points to the right and the left are caused by the double refraction of the spindle, being very marked at its ends. According to W. J. Schmidt a much stronger double refraction of the spindle may be observed in other cases, the fibres being lighted up over their whole length. The double refraction is positive, as would be expected from protein fibres. Schmidt emphasizes that the birefringence can not be caused by strain, but can only be due to intrinsic and morphic double refraction, i. e. to the presence of rod-shaped particles. This result is, however, not sufficient to explain the way in which the spindle is formed. Schmidt favors the conception that primary valence chains or micellae preformed in the cytoplasm are oriented, i. e. we would have a process similar to the formation of tactoids. It probably would not make an essential difference, if the units building up the spindle consisted of a newly formed substance. Apparently this structure does not allow one to account for the part played by the spindle in the movement of the chromosomes; though gel-like, the spindle seems to be very soft and this presents some difficulties, when trying to apply some obvious modes of explanation (*26*).

The structure of the heads of spermatozoa and of chromosomes may also be related to that of tactoids (*27*). The heads of spermatozoa consist of a mixture or a compound of proteins and nucleic acids. Provided that the heads are not spherical, but lengthened, rod-shaped, they are frequently found to show double refraction and mostly one which is negative, referring to an optical axis parallel to the long axis of the head. In Fig. 5 a photograph is shown of spermatozoa of Metrioptera

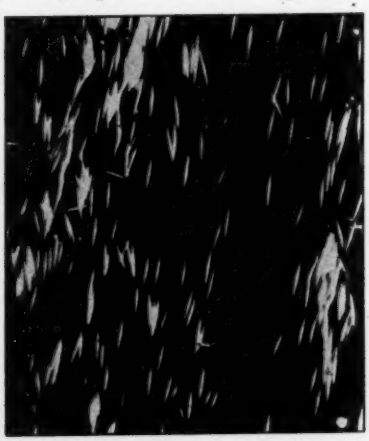


Fig. 5. Double refraction of the heads of spermatozoa of *Metrioptera roselii* (W. J. Schmidt).

roeselii. As this birefringence is not markedly changed by a change in the refractive index of the surrounding medium, it is an intrinsic double refraction. It is very probably caused by rod-shaped particles of nucleic acid being oriented with their long axes parallel to the axis of the spermatozoa head: it can be shown that stretched strips of a gel of α-thymonucleic acid or threads made artificially out of this substance are naturally negatively double refracting, the optical axis being parallel to the direction of strain or the long axis of the thread respectively. In some cases under suitable conditions, e. g. on swelling, the heads may become wholly or in part positively double refracting. This is explained as a morphic double refraction which would be positive because both the particles of nucleic acid and of proteins between them are rod-shaped and are oriented parallel to the long axis of the head. The entire behavior agrees well with the concept that we are dealing with a superposition of the intrinsic, negative double refraction of the nucleic acid and the morphic positive double refraction of rod-shaped particles oriented parallel to each other. The fact that the heads of many spermatozoa are not double refracting at all, can be readily explained by assuming that the negative birefringence is just compensated by the positive one.

The investigation of the very large, ribbon-shaped chromosomes in the salivary gland of Drosophila has proved that the nucleic acids are not distributed uniformly over the whole chromosome, but that they are distributed in segments standing perpendicularly to the long axis of the chromosome indicating the locus of the genes (28). It has also been shown that the segments always contain proteins (29) and there are strong indications that proteins are the backbone of the whole chromosome. This leads to the question (30): are proteins and nucleic

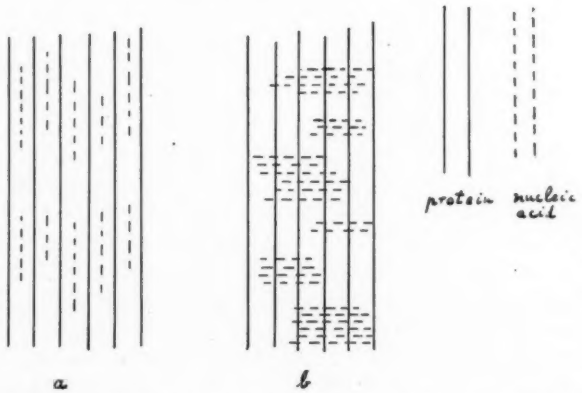


Fig. 6. Possible structures of chromosomes (A. Frey-Wyssling).

acids oriented parallel to each other in the segments like in the heads of the spermatozoa or are the fibres of proteins the warp, those of the nucleic acids the woof in this texture, as is assumed by Dr. D. M. Wrinch (31)? (cf. Fig. 6a and b). In case 6a the chromosomes might be both positively or negatively double refracting depending on the competition between the intrinsic double refraction of the nucleic acids and the positive morphic double refraction of the oriented rod-shaped particles; in case b they would always be positively double refracting, if at all. As was already mentioned above, chromosomes are frequently found to be birefringent, e. g. those of the eggs of sea-urchins (cf. Fig. 4); in the latter case the double refraction is positive, the long axis of the chromosome being parallel to that of the spindle. Unfortunately in this case it is not known how the segments are oriented in the chromosome. The ribbon-like chromosomes of Drosophila do not show birefringence. In other cases a spiral structure of the chromosomes causes difficulties, when trying to decide this question (32). Only an orientation according to 6a would agree with the structure of a tactoid.

One might think that a non-oriented coacervate of a number of colloidal particles, especially proteins and nucleic acids, in the "resting" nucleus is transformed into the well oriented structure of tactoids in some step of mitosis or of the formation of spermatozoa. There are, however, strong reasons to assume that this is not so, but that the chromosomes are present as such in the "resting" nucleus, although they are not visible, probably owing to a too high degree of hydration (33).

—As was mentioned above, the reproduction of macromolecules as in viruses will most likely in future have to be the next point of discussion, a process which is presumably one of autocatalysis. So far, colloid science can not help in making it better understood.—

Other cases of colloidal influence in development and growth.

The third group of processes to which we now turn have a factor in common in that they all deal with the development and growth of more complicated structures: with that of tissues and in tissues. Although the more chemical side of the problems in question, the influence of organizers, of growth hormones etc., is of major interest, colloidal factors, somewhat different from those discussed so far, also come into play. Some of these processes are related to the remarkable properties of gels. Gels unite to a certain extent the properties of liquids and solids. If not too concentrated, diffusion of small molecules and ions goes on in gels practically with the same rate as in true solutions; hence chemical reactions proceed in the same way; even coarse, microscopic particles of quartz etc. and droplets of oil migrate electrophoretically in a not too concentrated gel just as quickly as in a sol. On the other

hand, as in a solid, we have no convection currents which can cause disturbances; the products of chemical reactions occurring in the gel remain, where they are formed; there is no settling of precipitates. Furthermore, as was mentioned already, gels can be exposed to a strain which causes the particles, provided that they are not spherical, to be oriented, rod-shaped particles lying with their long axes parallel to the direction of the tension, and they may remain in their new position, when the gel, after having become harder, is released from its original tensions. If the particles of the gel are sufficiently small, uniform and have a refractive index sufficiently different from that of the surrounding medium, the gel becomes double refracting on stretching and remains so.

This orientation of particles of a gel leads to an interesting factor influencing the growth of cells, a phenomenon which can be joined rather naturally to the phenomena discussed in the preceding chapter. It was observed that in organisms and in tissue cultures cells preferred to grow in the lines of tension (cf. Fig. 7). But it was shown by Paul Weiss (34) that it is not actually the tension which is instrumental: if the culture medium, a membrane of blood plasma coagulated under stress on a triangular frame, is then removed from the frame and supported so that it is released from the original tensions, the cells of the tissue culture, nevertheless, grow out in the directions of the original tensions i. e. normally to the three sides of the triangle. The reason is

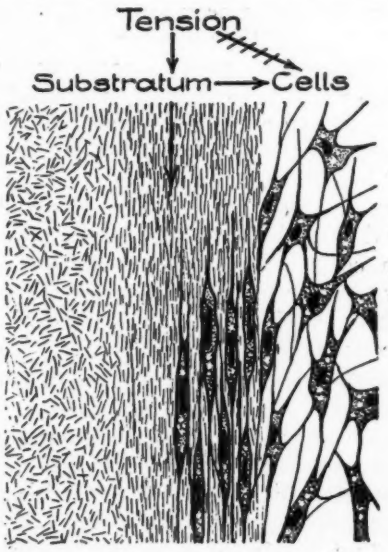


Fig. 7. Influence of oriented particles in gels upon the growth of cells (Paul Weiss).

that in these directions the rod-shaped particles of fibrin are markedly oriented. Now, cells apparently need a certain support for growing; growth is favored by solid surfaces, and the oriented fibrin particles of the coagulated plasma form, as it were, a pathway for the growing cells. The growth of nerves from neuroblasts in tissue cultures was shown to be influenced in the same way by oriented particles in the medium (35). In this case, it was further proved that the mechanical action can not be replaced by a chemical or an electric influence.

These results lead to a general principle (34) that the orientation of non-spherical particles in gel-like structures under the influence of stress, of folding etc. is essential for the arrangement of the cells in a growing organism; the oriented ultrastructure establishes, so to say, a pathway for growth.

In homogeneous liquid solutions convection currents, the settling of precipitates, intentional stirring make it impossible to split up the reaction into its primary processes and lead to results which represent, as it were, an average of the entire process. Not so in gels: if a series of processes is initiated by a substance diffusing from one side into a gel, where it may react with other substances, the results of these processes may be localized and differ markedly from point to point in the gel, depending on the concentration of the reacting substances etc. This gives in very general terms the reasons why the so-called Liesegang's patterns (36) are formed: many reactions which lead to a uniformly dispersed precipitate in homogeneous solution, produce the precipitate in characteristic striations, if they proceed in a gel. In some cases, the gel can be replaced by the narrow space between a glass and a watch-glass pressed upon it or by that of a thin capillary, where convection is reduced too. We do not know much about the mechanism—or probably mechanisms—of the formation of Liesegang's patterns, because it is difficult to decide, which of the many possible partial processes are actually involved. The following partial processes may occur: supersaturation and formation of nuclei, growth of nuclei, actual precipitation, coagulation of the precipitated particles, protective action of the gel as to coagulation, peptisation (or solution) of the precipitate by new substances formed in the chemical reactions going on. There are some specific points in the formation of Liesegang's patterns which have lead observers even to suppose some kind of wave mechanism (37).

It has frequently been assumed that the striated and dotted patterns in plants and animals or even the complicated patterns on the wings of lepidoptera are produced by processes of this kind (38). Not by a comparatively simple process as in an ordinary Liesegang precipitation, but it was assumed that a series of subsequent propagations of different substances diffusing through a complicated gel structure had occurred.

It has been shown that the pattern of a butterfly wing is indicated early in the pupa in relief, and that first in a later stage the final colors are called forth (39).

Obviously it is not easy to decide, whether Liesegang's phenomenon is important, particularly as we are so uncertain about its mechanism even in simple cases. Hence, as a rule, its significance in biological processes is not rated highly. I believe, on the other hand, that it ought not to be underrated, because the experimental conditions leading to Liesegang's patterns readily occur in growth and development.

Finally, the active part which may be played by water and aqueous solutions in the processes in question must be briefly discussed, since it always must be related in some way to colloidal phenomena, namely to osmosis or to swelling, two phenomena which perhaps in many cases do not allow a sharp discrimination.

Speaking of osmosis, I do not mean only the orthodox osmosis as it is observed with strictly semipermeable membranes leading to the theoretically well defined phenomenon of osmotic pressure. I include the large number of osmotic phenomena observed with membranes of complex permeability—membranes which let all substances present pass through, but with different velocity, or which are asymmetrical, allowing a substance to pass with ease from the one side, with difficulty from the other etc.;—under these conditions the final state is not characterized by a well defined osmotic pressure, but nevertheless, strong, dynamical osmotic effects may arise, as long as the solutions on both sides of the membrane have not adjusted themselves according to their final concentrations. We may then be dealing with abnormal osmosis which may be both positive, i. e. the liquid is transported, as in normal osmosis, from the more dilute solution to the more concentrated, or negative, when the transport occurs in the opposite direction, from the more concentrated solution to the more dilute one. Abnormal osmosis may be caused by electrosmosis (40); or with membranes, which are able to swell, it also may be due to a different rate of swelling on both sides of the membrane, it being a general rule that the liquid is transported from that side of the membrane which swells quicker to the other side (41). Abnormal osmosis may be quite as important as normal osmosis in biological phenomena.

There are processes of growth in plants which apparently are mainly produced by osmosis. Thus the growth of pollen tubes, the stretching of hypocotyls and roots have been found to be conditioned and favored by a certain acidity of the surrounding medium (42). The growth of pollen tubes, for instance, has an optimum at a pH of 6 to 6.2 with a maximum growth of 895 to 940 μ per hour. The rate also depends upon the nature of the anions. We are dealing most likely with a negative osmosis; whether it is due to electrosmosis or to different rates of

swelling or to both factors, has not yet been cleared up. Perhaps the possibility has not yet been sufficiently excluded that the acidity acts in an indirect way, causing, for instance a decrease in the resistance of the cell wall towards being stretched.

Wherever tissues are stretched or folded, we may have to take swelling into account. Unfortunately, swelling has also turned out to be a very involved phenomenon; it is probably the result of both a Donnan effect, i. e. an osmotic effect, and of hydration (43). That hydration comes into play, is proved by the fact that ions influence swelling according to the Hofmeister series: it is one of the few results concerning hydration established during the last years that the position of an ion in the Hofmeister series indicates its degree of hydration or, at least, its affinity to water, $Li^{\cdot}$ and F' or SO_4'' on the one end having a high affinity, $Cs^{\cdot}$ and I' or CNS' on the other having a low affinity (44). The rather puzzling fact that CNS' (and I') favor the swelling of proteins, cellulose etc. strongly, whereas SO_4'' decreases it, has been shown to be correlated to the marked positive adsorption of CNS' by the substances mentioned, whereas SO_4'' is negatively adsorbed (45), i. e. water is taken up more strongly than the sulphate, the latter, therefore, remaining in the solution. Hence it is readily understood, why SO_4'' decreases swelling. The increase caused by CNS' is explained by the fact that it is adsorbed on the protein surface in points which are not identical with the hydrophilic points of the protein; the hydration due to the CNS' is, therefore, added to the normal one of the protein.

The additive effect of the hydration of ions according to the Hofmeister series is probably complicated, already in medium concentrations, by a competitive, antagonistic effect between the ions: the small, strongly hydrophilic ions like $Li^{\cdot}$ and F' are apparently particularly effective, when associated with large, poorly hydrophilic ions like I' (and CNS') or $Cs^{\cdot}$ (46).

Concerning the hydration of hydrophilic colloids such as proteins, starch etc., our knowledge is still vague and fragmentary and the results obtained with different methods do not agree well. Theoretical conclusions also do not agree well with experimental results, perhaps with exception of the amount of water closely bound to proteins like gelatin and calculated under the assumption that each hydrophilic group of the proteins just binds one molecule of water (47).

Hence, it hardly may be hoped that we can say, where and how swelling can influence growth and development, till we know more about the details of swelling and the way it is influenced by ions and other substances.

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DISCUSSION REMARKS

TO HERBERT FREUNDLICH'S PAPER ON "COLLOIDAL CHEMISTRY OF DEVELOPMENT AND GROWTH"

By

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Growth consists of many coordinated events. Of these, Dr. Freundlich has invited intensive consideration of that group which concerns the formation of ultramicroscopic but macromolecular structures in growing cells. There is no guarantee that this aspect is more or less crucial than any other one in gaining insight into processes of growth and development.

1. What methods are available from the armamentarium of colloid chemistry, for the study of living cells that are growing and differen-

tiating? Severe restrictions follow from the desire to deal only with cells that survive observation. Localized regions, often representing growth zones, differ in measurable respects from non-growing regions.

Investigations have been made of electrical charges upon visible particles, Brownian movements, viscosity, elasticity, tensions, swelling, arrangements of ultramicroscopic particles, birefringence, X-ray diffraction patterns, visible phases, and some others. In respect to several of these properties some "young" protoplasms have been shown to differ from "old" ones of the same species. It may be surmised from diverse materials that young cells have fewer permanent structures, are less viscous, and have lower tensions at their surfaces. Those properties are often said to characterize states of less differentiation.

In multicellular individuals it is more difficult to find age-distinctions within cells than with respect to the ways in which cells are arranged and interact. For, the cohesions, shapes, metabolisms, and excitations of cells also have colloidal aspects.

Most of the information presented as colloid chemistry of biological systems concerns either models thought to resemble protoplasms, or once-living substances. Studies upon such may be useful preliminaries toward the description of living systems. Recognizing that vital activities are proceeding in delicately maintained steady states, "gentle" methods of probing protoplasms are of course preferred. No criteria other than arbitrary tests of survival are generally agreed upon in limiting the definition of "living systems." The usefulness of X-ray diffraction patterns, for instance, as indications of living states is of debated significance.

2. Single cells that have been found favorable for the study of localized ultrastructures during growth are: axons, myoblasts, sea-urchin eggs, stamen-hair cells, sporophores, and coleoptiles. What has been learned in them by the use of polarized light as outlined by Dr. Freundlich?

The primary walls of cylindrical cells are laid down sub-apically as transverse or tangential chains of micelles. That is true whether in axons (proteins, Schmitt, 1936 and Taylor, 1940), in sporophores (chitin, Heyn, 1936 and Castle, 1938), or coleoptiles (cellulose, Thimann, 1938 and Wuhrmann-Meyer, 1939). The fact leads to a picture of growth (elongation) by intussusception in one dimension; for it occurs by the intercalation of new chains in side by side arrangement, as when logs are made into corduroy road. The rate at which micellar chains are added can be computed, and the fact that no visible jerks occur in the elongation has been recognized (Castle, 1940). Similar depositions occur during growth in two and three dimensions; the direction in which micelles are oriented in superficial or in solid networks is the counterpart of the kind of growth.

In some kinds of cells crystalline macromolecules are deposited only on the surfaces; in others they are also present in deeper layers. Whether they are deposited reversibly like automobiles in a parking lot is uncertain. Whether during deposition only crystallization occurs, or certain syntheses as well, has not been ascertained. Possibly the orientation is gradual after the micelles have materialized, as Iterson, 1937 suggests for stamen-hair cells. That the size and shape of the cell is set by the conformation of its surface is not to be inferred. There is no information concerning the means by which the rate of steady deposition is fixed; that appears to be a major physiological question concerning growth and development. The progression in space and in time of the local differences in rate, points to a pattern of several dependent variables.

3. Growth zones, whether subcellular or multicellular, are the sites of coordinated physiological processes. The known processes are peculiar to those regions only quantitively. Electrical negativity, rapid respiration, salt accumulation, protein accumulation, and streaming of cell contents are recognized correlatives of growth (Steward, 1937). These events help to define the sort of setting in which colloidal architecture is being laid down.

Often it is supposed that patterns of development may be detected earlier in the invisible structures than in visible ones. This surreptitious preformationism is not particularly supported by present knowledge. An invisible pattern may equally well exist without any material counterpart.

Nevertheless, notions of colloid chemistry suggest concrete and testable hypotheses concerning development. Instead of a cell as a sack of chaotic suspension, a cell is conceived as an ordered arrangement of materials at all levels of particulate size. Under the influences of certain preceding events, continuity of energy transformations and of ultrastructures provides a net local accumulation beyond the steady turnover of living. In so far as accumulated materials become colloidal they can be measured and identified in each kind and rate of growth and development.

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CHEMICAL FACTORS OF PLANT GROWTH

By

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The chemical factors which control, regulate, or condition growth in plants can be divided into three main categories. First of all, all organisms require for their maintenance a supply of suitable inorganic building materials such as nitrogen, potassium, sulfur, etc., and carbon in some form as a source of energy. These elements we regard as nutritional substances because they are used in relatively large amounts and are the building blocks out of which protoplasm constructs itself. In a broad sense, nutritive materials are growth regulators. This is less clear for microorganisms than for higher plants where nitrogen or potassium deficiencies may, for example, produce characteristic architectural anomalies. In the second place, many if not all plants require certain other inorganic substances which are growth-regulatory but which are not metabolized as are the nutritive materials just mentioned. These are effective when present in the culture medium in very minute amounts. Schopfer (1939) refers to them as pseudo-growth factors. Hoagland (1937) calls them micro-elements. Zinc, manganese, thallium, boron, molybdenum, copper, vanadium, etc. are examples. In general, they have a stimulatory effect on the growth of organisms which is transitory. Being elements, they of course originate outside the organism. In the third place, plants require certain specific organic substances many of which are normally produced in the living protoplasm; such substances are active when present in very small amounts, and are essential for the continued growth and reproduction of the organism. These substances are the *growth factors* (accessory substances) of microorganisms, and the *hormones* (growth hormone, growth substances, physiologically active substance, auxin, etc.) of the higher plants. Huxley (1935) proposed the inclusive term "activators" for chemical substances produced by the organism which exert specific functions in regard to correlation or differentiation. As a biological definition, Huxley's term is brief and descriptive for a host of physiologically active substances the chemical roles of which we are for the most part at least temporarily ignorant. It is equally applicable to all kinds of organisms. It fails, however, as biological terms commonly do, to help us understand the part played by such substances in metabolism.

Since growth factors or accessory substances are produced in greatest amounts during the synthesis of protoplasm, development control is chiefly characteristic of young or potentially young organisms, or younger parts of older organisms. Hence, it is to these that we must

turn our attention if we are to examine the effects of chemical factors upon growth. The discussion will center chiefly around certain microorganisms (fungi) and higher green plants.

MICROORGANISMS

Microorganisms serve well for growth factor studies for a number of reasons. They reproduce rapidly, they are readily cultured, and they generally require attention only at the beginning and end of an experiment. Most important of all, some species and strains are unable to produce amounts of certain growth factors adequate for their own optimal development. These deficiencies must be supplied from without. It is through supplying such accessory substances to organisms deficient in the capacity to synthesize them in adequate amounts, that our knowledge of this subject has advanced.

Detection of unknown growth factors.

Two general approaches are commonly employed for determining the growth factor requirements of a microorganism:

1. A suitable culture medium may be chemically treated so as to destroy or remove certain growth factors. Regarding this treated medium as optimal except for growth factor needs, the next steps involve identification of the previously removed unknown substances.

2. A synthetic nutrient medium, presumably optimal for the growth of the organism (except for growth factors) is supplemented with plant or animal extracts which enable the organism to grow. The growth factors may then be isolated and identified. More recently, chemically pure substances, suspected of growth factor activity (or mixtures of several of them), are added to the synthetic medium until good growth occurs.

It is important, as Schopfer (1939) points out, that: (a) physical and chemical properties of the culture medium which would check growth of the organism be eliminated; (b) the unknown substance suspected of being a growth factor must maintain its effectiveness in very high dilution when added to the culture medium; (c) all sources of error must be eliminated, such as contamination of the medium with growth factors.

Koser and Saunders (1938, p. 102) point out briefly some of the difficulties attending sterilization of the fractions to be tested, basic medium for the tests, size of inoculum, etc.

Various methods of assay have been employed: for yeast, turbidity of the culture medium in relation to that of the control, is most commonly used; for molds, dry weight of the mycelium; for bacteria, acidity produced.

Identity of some of the growth factors.

Vitamin B_1, or thiamin, is thus far the best known growth factor for microorganisms. It is also a hormone for higher plants. Its molecule is characterized by two ring structures, pyrimidine and thiazole (Williams, 1938), and for certain organisms one or the other or both of these components are quite as effective growth factors as the intact thiamin molecule. Table I lists a number of organisms known to require thiamin or its components for satisfactory growth. The list is only a partial one.

TABLE I

PARTIAL LIST OF DIVERSE ORGANISMS REQUIRING THIAMIN OR ITS COMPONENTS FOR SATISFACTORY GROWTH

Organism	Growth factor requirement				Authority
	Thiamin (intact molecule)	Pyrimidine and Thiazol	Pyrimidine	Thiazol	
Protozoa					
Polytomella caeca	x				Lwoff and Dusi, 1937.
Strigomonas oncopelti	x				Lwoff, 1937.
Glaucoma piriformis	x				Lwoff and Lwoff, 1937.
Bacteria					
Propionibacterium pentosaceum	x				Tatum, Wood and Peterson, 1936.
Staphylococcus aureus	x				Knight, 1937.
Phycomycetes					
Phycomyces Blakesleeanus	x (either)	x			Schopfer, 1934. Robbins and Kavanagh, 1938
Mucor Ramannianus	x (either)	x	or	x (alone)	Muller and Schopfer, 1937.
Phytophthora fagopyri		x (either)	x		Robbins and Kavanagh, 1938.
Ascomycetes					
Saccharomyces cerevisiae	x				Schopfer, 1939.
Helvella infula	x				Fries, 1938.
Basidiomycetes					
Ustilago violacea	x	x			Schopfer and Blumer, 1938.
Polyporus adustus	x	x	x		Fries, 1938. Schopfer and Blumer, 1938.
Fomes ignarius	x				Noecker, 1938.
Fungi imperfecti					
Rhodotorula rubra	x (fair)	x (good)	x (good)		Schopfer, 1937.

The concentrations needed to satisfy growth factor requirements vary with the organism, but Schopfer (1939) has calculated that 0.0004γ of thiamin will give a 1 mg. increase in the dry weight of *Ustilago violacea*, whereas 0.005γ is needed for a 1 mg. increase in the dry weight of *Phycomyces Blakesleeanus* or *Rhodotorula rubra*. The best growth of *Phycomyces* occurs when thiamin is present in the culture solution in a concentration of approximately 2γ/10 cc.

Substituted pyrimidines and thiazoles have shown a high degree of specificity, and only a few are reported as effective substitutes for the original component (Knight and McIlwain, 1938, etc.)

The old bios complex is now known to be a mixture of at least *i*-inositol (Eastcott,, 1928), biotin (Kögl and Tonnis, 1936) and pantothenic acid (Williams *et al,* 1931, 1933, etc.). Other well-known growth factors are hematin, nicotinic acid, riboflavin, pimelic acid, uracil, glutamine, vitamin B_6, etc.

Many organisms (including some of those listed in Table I) lack the capacity to synthesize several growth factors, at least in amounts needed for optimal growth, and these must be supplied through the culture medium. Here are a few examples (cf. Schopfer, 1939):

Nematospora gossypii	*i*-inositol + biotin (thiamin is a cofactor?)
Streptobacterium plantarum (Möller, 1939)	vitamin B_6, nicotinic acid, biotin, pantothenic acid
Saccharomyces cerevisiae	thiamin + *i*-inositol + biotin + β-alanine
Strigomonas fasciculata	hematin + ascorbic acid
Corynebacterium diphtheriae	β-alanine (nicotinic and pimelic acids are cofactors)

Specific roles of growth factors.

What is the role of these substances in the metabolism of microorganisms? In brief, those we know most about seem to be precursors of coenzymes, parts of coenzyme complexes, or coenzymes themselves, and are largely concerned with cell oxidations. The pyrophosphoric acid ester of thiamin can, for example, act as a cocarboxylase; nicotinamide as a component of cozymase; riboflavin as a component of the "yellow enzyme" of Warburg and Christian, etc. (see Euler, 1938; Schopfer, 1939; Williams, 1938).

A basis for symbiosis and parasitism.

From the foregoing it will be clear that some microorganisms can synthesize growth factors which others cannot. Thus, if two different organisms are growing together and each produces a growth factor helpful to the other, the basis is provided for a symbiotic relationship. Kögl and Fries (1937) established such a relationship between two fungi growing together in culture, *Polyporus adustus* (Basidiomycete) and *Nematospora gossypii* (Ascomycete). The former produces biotin, needed by the latter for satisfactory growth, while *Nematospora* produces thiamin, needed by *Polyporus* for satisfactory growth. Result: symbiosis *in vitro.* This work provides an important lead to a fuller understanding of symbiosis and of parasitism (also see Robbins, 1939).

Symbiosis in relation to form and development.

An opportunity arises now to speculate a bit on the extent to which

growth factors may affect form and development of plant bodies.

The lichens represent a group of plants known to all biologists. They are the professional symbionts of the plant kingdom, classic examples of compound organisms which exhibit symbiosis. One of the component organisms, the alga, synthesizes food which it delivers to the fungus component, while the latter supplies minerals and water to the alga. This relationship has been explained in the past as a perfectly straightforward nutrient partnership. The situation now seems less simple: It is undoubtedly *more* than a nutritive bond which holds the alga and fungus together, and produces an organism with new characteristics, an organism which is more than the sum of its parts. So stable is this relationship that the resulting plant bodies require generic and specific names.

Lichens present a natural but as yet untouched source of experimental material for the study of growth factors. Nowhere else in the plant kingdom, so far as I know, do two completely different organisms live in such intimate relationship and produce specific new physical forms. There are no genes for lichens. What, then, explains the new emergent form? Genetics is out. Environment is out, except in so far as each component is part of the environment of the other component. Nutrition offers limited possibilities of explaining the mode of their existence, but contributes nothing to an explanation of the new physical form assumed. Thus the lowly lichen appears to present a growth factor problem, which, if properly explored, may shed new light on specific organic substances and their relation to control of form and development.

HIGHER PLANTS

Now to go back to thiamin, the best known of the growth factors of microorganisms. It is also well known for its importance in the growth of higher plants. The first experimental work in this connection was done on excised pea embryos (Kögl and Haagen-Smit, 1936), and more particularly on root cultures by White, Robbins and Bartley, and Bonner, all working independently. White (1937) reported on excised tomato roots, Robbins and Bartley (1937) also on excised tomato roots, and Bonner (1937) on excised pea roots.

The question of definition immediately arises . . . . . is thiamin a "growth factor" for higher plants, a "growth hormone", or both: as related to growth of excised roots living in culture, thiamin is a *growth factor* (according to definition), i. e., it is a specific organic substance necessary for root growth which must be supplied from without. The excised root in culture cannot make its own thiamin. But in intact higher plants, thiamin is a hormone (according to definition), i. e., it is synthesized in the green parts (above ground), and from there moves

down to the roots, where it is necessary for growth. Obviously, there is no difference in the physiological rôle of thiamin in roots, whether they grow *in vivo* or *in vitro*. Thus the definitions break down. Thiamin and probably most other physiologically active substances are "activators" in a broad sense, and except from the point of view of correlation, it seems unprofitable longer to be concerned with terminology. Chemical advances will make specific activities of such substances clear, and that is what will lead us nearer to the goal of growth control.

Naturally produced growth substances.

The presently known, naturally occurring "growth substances" in higher plants are no longer new: auxentriolic, auxenolonic, and 3-indole acetic acid. Their existence was first established through study of the influence of unilateral light and gravity on tropistic responses (cf. Boysen Jensen *et al*, 1936; Went and Thimann, 1937). Thus far the chemical identification of this class of naturally occurring substances has been limited chiefly to the work of Kögl and his associates.

No simple test has been devised to distinguish chemically between the substances mentioned (in the low concentrations in which they naturally occur), but the *Avena* test method devised by Went (1928) is widely used for determining their biological activity. In normally occurring concentrations they are substances which promote or retard cell extension (concentrations favorable to stem elongation are inhibitory to root elongation), and are commonly referred to as growth substances, growth hormones, or auxins. Details regarding methods of assay, etc., are treated at length by Boysen Jensen (1936) and Went and Thimann (1937).

Occurrence and production.

A word about their occurrence: Auxins are known to be present in plants representative of practically all groups, and they are known to occur in all plant organs. They are also known to be present in human urine, and in numerous animal tissues, including carcinoma. As for their natural production: present evidence indicates that in higher plants, centers of auxin production are the regions where new protoplasm is being synthesized. This means the embryonic parts of plants, the root tips, stem tips, embryonic leaves, and to a less extent the cambium, for these regions are, in a sense, the protoplasm "factories." High available nitrogen in the soil in which plants are growing brings about very rapid growth of stem tips and associated with this is higher-than-normal auxin production (Avery, Burkholder, and Creighton, 1937).

A number of physiologically active substances of the auxin category have been synthesized, although most of these are not now known to

occur in plants. 3-indoleacetic acid ("heteroauxin") is the most familiar of these; though now synthesized in pure form, it was first obtained from human urine for auxin studies, and later from yeast, and from *Rhizopus* cultures (cf. Boysen Jensen *et al*, 1936). It is reported to have no effect on the growth of yeast, or on *Rhizopus*, even though produced by them. Among other chemically pure substances active as auxins, are indole butyric and α-naphthalene-acetic acids (Zimmerman and Wilcoxon, 1935). These are highly effective in the *Avena* and other tests (Avery, Burkholder, and Creighton, 1937; Zimmerman and Hitchcock, 1937), as are numerous of their derivatives.

Growth substances in relation to normal growth.

What evidence is there for auxins being agents which exercise normal developmental control, at least in higher plants? The best we can say now is that there are numerous correlations between normal growth and the occurrence of an auxin or auxins; causal relationships are as yet not proved beyond question. Only relatively low concentrations of auxin are involved here, perhaps the equivalent of 5-50γ per liter of indoleacetic acid.

It has been shown in the tobacco leaf (Avery, 1935) that there is an auxin gradient from tip to base of leaf; the concentration increases toward the base, presumably due to accumulation in the midrib of the leaf, and this higher concentration is correlated with greater longitudinal growth ("polarized" growth) of the midrib in this region. Inasmuch as the application of lanolin containing indoleacetic acid (to large veins of the leaf) brings about a growth response, it may be concluded that growth hormones are the agents responsible for promoting the normal growth in length of the midrib and larger lateral veins.

Goodwin (1937), working on leaves of golden rod, has shown that in the development of the leaf the highest auxin concentrations are nearly coincident with the period of most rapid growth, and in one species he reported that in the young basal rosette of leaves, only one leaf is elongating rapidly at a time. This retarding effect on the succeeding leaves was correlated with high auxin production in the rapidly elongating leaf; if the latter were removed, growth in the succeeding leaves speeded up, but indoleacetic acid applied to the cut stub of the leaf (in the proper concentrations) caused retardation of the succeeding leaves, comparable to that caused by the intact leaf.

Studying auxin in relation to the branching habit of two species of aster, Delisle (1937) concluded that the extent of development of secondary branches is correlated with the amount of auxin produced in

the tip of the main stem, and to a lesser extent that produced in the young developing leaves. This represents a naturally occurring example of the behaviour induced experimentally by Skoog and Thimann (1934) when they showed that if the growing tip of a plant were removed, and auxin (in agar) applied in its place, the effect was to inhibit, at least for a time, the development of the lateral buds.

That dwarf varieties of plants may get their dwarfness from auxin destruction is indicated by the work of van Overbeek (1938). He reports that seedlings of the dwarf maize variety *nana* produce less auxin than the normals (at least for a time in their development), respond less to applied auxin, and that their tissue is more active than normal in the destruction of auxin.

There is a variety of maize known as *lazy* which has a prostrate habit of growth, i. e., it is indifferent to gravity during most of its development, and simply lies on the ground. Normal varieties, on the other hand, tend to grow upright if placed in a horizontal position, due apparently to the fact that auxin accumulates on the lower side of the stem, speeds growth there, and causes the stem to bend upward away from the force of gravity. But in *lazy* maize, auxin distribution is abnormal, i. e., more of it is found in the upper than in the lower half of the stem (van Overbeek, 1938; Shafer, 1939), thus suggesting that abnormal growth habit in many plants may find its explanation in abnormal auxin distribution in the plant.

It has been known for a long time that when tree growth starts each spring, growth in diameter (from the cambium) is initiated in the twigs adjacent to the terminal buds, and travels downward in the stem. This "cambial stimulus", as it has been called, became understandable when Snow (1935) applied auxentriolic acid, and indoleacetic acid, in concentrations of 1 or 2 ppm. to decapitated young sunflower plants, and found that cell division in the cambium followed. Avery, Burkholder, and Creighton (1937), studying horse chestnut and apple trees, found that auxin concentration in buds increased rapidly prior to the time of their bursting in spring, and that auxin could later be detected at successively lower levels in the twigs. The auxin, in its downward movement, always preceded cambial activity, and this, in the light of Snow's work, led to the conclusion that it probably acts as a cambial stimulus.

In the embryonic stems of pumpkin, squash, and watermelon, there is produced a small outgrowth which is effective in holding the seed coats open during germination. It appears that auxin from the inner seedcoat is the agent responsible for this "peg", and LaRue (1939) concludes that it is the only plant structure known which is entirely dependent on a growth hormone for its existence.

When placed in darkness the younger portions of green plants grow very rapidly. Their stems elongate abnormally and become spindly in a remarkably short time. The plant parts thus affected are said to be "etiolated", and the idea of a possible relation of auxin to etiolation goes back to the work of Thimann and Skoog (1934), who showed that a given amount of auxin is much more effective in growth of a stem in darkness than it is in light. More recently, Larsen (1939) has extracted an etiolation substance from pea plants grown in darkness, but regards it as probably identical with one of the already known naturally occurring auxins.

Thus we see that normal growth expression of one sort or another is correlated with the presence of physiologically high concentrations of auxins, but in most cases it is by no means certain whether the relationship is one of cause or effect. As new methods of attack are developed, much of the past work can be repeated with profit.

There are many other problems awaiting investigation from the hormone point of view. Here are two which have had preliminary study, but have thus far given no very helpful results: The significance of auxin in germination of seeds is an unsettled problem, but has been treated at some length by Voss (1939). One thing seems clear, even though causal explanations must await further evidence: dormant maize seeds have a good supply of auxin which gradually disappears during their germination (Avery, Creighton, and Shalucha, 1940). It is not yet clear whether this disappearance means use by the developing embryo, or destruction.

One possible approach to a physiological explanation of hybrid vigor is through a study of auxin content of hybrids and parents. A preliminary attack on the problem in seeds of inbred and hybrid lines of maize has shown auxin yield (per gram of endosperm) in hybrids generally to be intermediate between that of the parents (Avery, Creighton, and Shalucha, 1939).

Responses of tissues to synthetic auxins of known chemical structure.

The evidence regarding tissue responses rests on the results of applying high concentrations of the chemicals to various parts of higher plants. In many of the experiments described, 3-indoleacetic acid has been applied to cut or intact tissues in concentrations as high as 2 and 3 per cent, in lanolin. This is probably from a few to several hundred thousand times higher than concentrations occurring normally in vegetative tissues, but there is as yet no evidence to indicate that more than a small part of this actually enters the tissues. Here are some of the results:

Propagation of plants by cuttings is a familiar horticultural prac-

tice. Many species root slowly, sparsely, or only with the greatest difficulty under ordinary circumstances, but if indoleacetic, indolebutyric, or α-naphthalene-acetic acids (etc.) are supplied to cuttings in the proper concentrations, vigorous rooting usually occurs, and in a much shorter time than in the untreated controls. Such treatment of cuttings is now a widespread practice among nurserymen and others. At the inception of this work 6 or 7 years ago, it was thought that the active substance with a specific root-forming hormone (an "organ-forming substance"). It soon became clear that this substance was identical with indoleacetic acid . . . . which was observed to bring about numerous and sundry responses in plants (cf. Growth Hormones in Plants, and Phytohormones). We now know that a considerable number of synthetic organic compounds are effective agents in encouraging the rooting response. Stuart and Marth (1938) found that treated cuttings accumulated carbohydrates in the region where rooting was to occur (but prior to its occurrence), thus indicating that auxins may be important, at least in high concentrations, as mobilizers of food materials (Stuart, 1938).

Galls similar to the crown gall caused by *Phytomonas tumefaciens* have been produced in bean and other species by Brown and Gardner (1936). Indoleacetic acid was the chemically pure gall-producing substance used, and an unidentified substance derived from cultures of *Phytomonas* was also found to produce galls. Link, Wilcox and Link (1937) report similar findings. And LaRue (1936) showed that indoleacetic acid could act as an intumescence-producing agent in poplar leaves. Thimann (1936) reported that indoleacetic acid probably is also the nodule-producing substance in roots of *Pisum.*

That all tissues are not equally responsive to applied auxins was the conclusion of Kraus, Brown and Hamner (1936), who placed indoleacetic acid in lanolin on the cut ends of young bean stems, and made histological observations on material prepared at 6 hour intervals over a week's time. The most striking response was a speeding up of nuclear division, such divisions often resulting in multinucleate cells. Cambium cells were quickly stimulated to divide, as Snow had found for lower concentrations of auxin; endodermis and phloem cells also responded readily by undergoing division; pith and cortical cells were less active, etc.

Using the same general method of Kraus *et al*, Greenleaf (1938) applied 1 per cent indoleacetic acid to decapitated *Nicotiana.* The result was good callus formation, multinucleate cells, and a large number of adventitious shoots approximately 14 per cent of which were tetraploid and a few octoploid. The same treatment of decapitated tomato plants resulted in no shoots whatever.

Continuing the work of Kraus and collaborators, but on tomato, Borthwick, Hamner, and Parker (1937) observed callus production with 3 per cent indoleacetic acid in lanolin, but concluded that the extent of the response, tissue for tissue, was less in tomato than in the bean; also that the usual development of tissues is altered but no new types were differentiated . . . . . such differences as existed were of degree rather than of kind. Microchemical studies showed that in the treated regions proteins increased and starch decreased.

The same general types of callus formation were reported from treating decapitated young bean stems with α-naphthaleneacetic, indolebutyric, and indoleacetic acids (Hamner and Kraus, 1937); there were "appreciable differences in the degree or intensity of responses of the several tissues in relation to these specific substances."

Observing the application of indoleacetic acid to intact young bean plants led Mitchell and Martin (1937) to conclude that it profoundly affects transport of materials, and indeed, Mitchell and Hamner (1938) showed that fresh and dry weights of parts of the plants near point of treatment were greater than in the controls. Subsequently, Stuart (1938) reported a large shift of nitrogen and carbohydrates from leaves, etc., of young plants to regions treated with 0.01 per cent indoleacetic acid. Kraus and Mitchell (1939) have carried forward this work on mobilization, using naphthaleneacetamide, and subsequent studies show (Mitchell and Stewart, 1939) that the latter compound induces mobilization of solid materials just as does napthaleneacetic acid, but causes less cellular proliferation than the latter.

In connection with the subsequent movement of a temporarily deposited insoluble form of nitrogen, Mitchell and Stuart (1939) state that "it is evident that the treatment stimulated the proteolytic enzymatic activity of the cuttings". There is evidence, as yet circumstantial, regarding high peptidase activity in cells with high auxin concentrations (Avery and Linderstrøm-Lang, 1940).

Beal reports (1938) different responses in different species of lily, as a result of applying indoleacetic acid to decapitated young stems. In *L. Harrisii,* 1 to 3 buds were induced to develop in the axils of each of the upper two or three leaves, although neither buds nor bud primordia were present at the time of treatment. Bud induction in this instance is just the opposite of the observation of bud suppression in another species (reported by Thimann and Skoog, 1934). In two other species of lily, however, no buds developed after treatment, though roots appeared for some distance below the point of application of the acid.

Other equally interesting though probably less important effects resulting from the application of auxins to plants, have been noted.

Perhaps one other is worth mentioning here. Leaves, flowers and fruits, as all are aware, tend to fall from the plant at one time or another. The mechanism of this leaf, flower or fruit fall resides in a definite plate of cells located at the end of the leaf or fruit stalk, referred to as the "abscission layer." We now know that abscission can be delayed (LaRue, 1936) or prevented by the application of indoleacetic or naphthaleneacetic acids to leaves, fruits, etc., at the proper time and in the right concentrations, and this discovery is making possible many new horticultural practices, including the production of seedless fruits (Gardner and Marth, 1937; Gustafson, 1938, etc.).

Thus the morphogenetic action of a pure chemical substance, such as indoleacetic acid, is seen to be exceedingly various. Whatever specificity-of-response there may be resides not in the substance applied, but in the tissues of the different species. As Hamner (1938) puts it, no single tissue or tissue system responds either quantitatively or qualitatively the same throughout the various types investigated. In a given species or variety, some tissues are more sensitive to stimulation than others, and accordingly give a greater response. The case is analagous to the coenzyme-enzyme relationship, where a single substance is known to act as a coenzyme for a number of enzymes. The specificity of the activity of the combination resides in the enzyme (Oppenheimer and Stern, 1939).

The question now arises, what is the mechanism of auxin action? We do not now know, but here are some of the more important lines of evidence, and a few suggestions: The facts regarding auxins and mobilization of materials have already been given. There is a parallel between the experimental work described and the situation existing in normal plants. Although the normal concentration of auxin in plants is but a small fraction of the concentrations which must be applied to get effects, it is likely that relatively small amounts of such applied auxins actually enter the tissues. If this is the case, the experimental work bears an important relationship to normal mobilization of materials in growing parts of plants. For example, the higher auxin concentrations characteristic of regions of protoplasm synthesis (embryonic regions such as stem tips) very likely play an important part in mobilizing materials (i. e., attracting food substances to such regions).

But whatever gross qualities auxins have as mobilizing substances, their specific physiological role or roles remains to be demonstrated. Suggestive evidence is to be found in the work of Sweeney and Thimann (1938) on the effect of auxins on protoplasm streaming. Concentrations of 0.01 mg. per liter of indoleacetic acid are reported as increasing the rate of streaming of protoplasm in *Avena* coleptile tissue,

but the influence is short-lived. In the presence of 1 per cent fructose, however, streaming is maintained at the accelerated rate for several hours. The results indicate that indoleacetic acid accelerates oxygen consumption (though duBuy and Olsen, 1940, have not been able to demonstrate this), which presumably controls the rate of streaming, and the latter is intimately related to growth. If established beyond question, it would definitely place the activity of indoleacetic acid in the chain of respiratory processes, probably as an activator of enzymes, or component of one or more enzyme systems. Given these facts . . . if such they turn out to be . . . we would at once understand the role of auxins as mobilizers. In the light of our information about growth factors for microorganisms, an early solution of the problem in higher plants is indicated along these lines.

There appears also to be a relationship between auxin and certain minor elements in the nutrition of higher plants: Eaton (1940) has shown that indoleacetic acid can to some extent replace boron as an essential element for the growth of cotton plants. If the foregoing suppositions regarding enzyme activation, etc., materialize, Eaton's work should tie in very well.

Other substances affecting higher plants: wound hormone, sex hormone, leaf growth hormone, and flower hormone.

Still other physiologically active substances have been demonstrated in higher plants: A substance called traumatic acid, a dibasic acid, has been isolated by English, Bonner and Haagen-Smit (1939) from plant tissues. It is reported to be capable of inducing cell division and extension in parenchymatous cells from the inside of the bean pod, and in the parenchyma of the potato tuber. The authors regard it as a "wound" hormone in the light of earlier suggestive studies by others.

Raper (1939) reports finding a complex of diffusable substances which initiate the development of sex organs in *Achlya ambisexualis* and *A. bisexualis* (heterothallic phycomycetes). The female mycelium apparently secretes a substance which diffuses through the liquid substrate to the male mycelium and induces male sex organs to develop; these, in turn, secrete a second substance which diffuses to the female mycelium where it induces the development of female sex organs. The female organs then secrete yet another substance which stimulates direction of growth of the male sex organs, and the latter later secrete a fourth substance which apparently stimulates delimitation of the female organs. The author regards these substances as "sex hormones", though their diffusion through the substrate is not within the present definition of a hormone (another instance of the breakdown of definitions). In any case, the first two substances Raper reports seem to be

specific sex substances, and as far as the present evidence goes, they may be regarded as the only substances which might qualify as the long-sought after "organ forming substances", if indeed such there be. There is evidence (Moewus, 1938) for carotinoids acting as sex determining substances in several species of algae, but this cannot be elaborated upon here.

It has been pointed out that auxin seems to be a causal agent in the length-growth of the midrib and larger lateral veins of the tobacco leaf. Bonner, Haagen-Smit and Went (1939) now give us preliminary evidence for the existence of a substance which favors growth of the leaf lamina, presumably the mesophyll of the intervenous portions of the leaf. The chemical isolation of such a substance would be a boon to our understanding of "plate" meristems (Avery, 1933), which are the forerunners of the large thin sheets of tissue which characterize leaves.

Perhaps the least satisfactory evidence to date of the many relatively recently reported new substances, is that for the induction of flowering in higher plants. Numerous studies have been published, but nothing seems sufficiently definite yet to warrant inclusion here.

SUMMARY

Living protoplasm normally makes its own growth factors ("accessory substances", or "activators"), hormones, etc., which are discussed here under the title of chemical factors of plant growth. Certain microorganisms, however, do not possess the ability to synthesize optimal amounts of these growth factors, and it is by supplying such deficient substances through the culture medium that we have learned about the necessity for them. Their roles in symbiosis and parasitism, as well as in normal growth, are becoming increasingly clear. Some of the substances in question (thiamin, nicotinic acid, etc.) are known to act as coenzymes or parts of coenzyme complexes.

As regards higher plants, it is shown that physiologically active substances (naturally occurring as well as synthetic) are capable of influencing form, as well as inducing a variety of morphogenetic effects. Specificity of response appears to reside in the protoplasm of the species. In general, these substances act as mobilizing agents when present in fairly high concentrations, but the specific means by which such mobilization is accomplished is not yet known. In the light of our information about growth factors for microorganisms, and from preliminary work on higher plants, an early determination of their role is indicated . . . they probably act as activators of enzymes, or components of enzyme systems.

The status of wound, sex, leaf growth and flower hormones is discussed briefly.

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DISCUSSION REMARKS

To G. S. Avery's Paper On "Chemical Factors Of Plant Growth"

By

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The principal interest of chemical growth factors for the physiologist lies in the possibility they offer of a tool to elucidate the process of growth. Dr. Avery has shown how the knowledge of auxin production and movement can provide an explanation of observed morphological phenomena. A field of special interest which might also be mentioned in this connection is the extension of the auxin work into pathology, where attempts have been made to consider the phenomena of galls and nodules in terms of excess local auxin formation.

There are two important difficulties in the study of auxin action, and these difficulties are not confined to plant hormones either. The first is that of *delayed action*. For example, seeds treated with auxin are slightly inhibited in their growth at first, but subsequently show a gradual acceleration, so that their final growth is larger and the yield of seed may even be (in the case of oats) increased by 30 or 40%. This result takes place many weeks or even months after the initial treatment, which perhaps lasted 24 hours only. Such phenomena are very hard to explain.

The second is of even more general significance. I refer to the phenomenon of *tissue specificity*, that is, the different effects which the same substance may produce in different tissues. Thus the oestrogens give rise to rapid growth in uterine tissues but not elsewhere. Auxins, at 10^{-10} molal, may promote the growth of roots, while higher concentrations only inhibit, yet in shoots the promotion of growth has its optimum concentration at about 10^{-4} molal; so that a given treatment with auxin may promote the growth of one part and inhibit that of another.

Perhaps the most extreme case of this differential response of different tissues is the one recently brought to light in the analysis of the behavior of slit stems to auxin. If elongating stems or similar organs are slit longitudinally and immersed in an auxin solution they curve inward. Direct growth measurements show that this is due to the outside of the stem growing more than the inside. Both are exposed to the same auxin concentration, and explanations based on the idea that auxin does not reach or enter the inner (wounded) side could be disproved. After much argument we have, I think, succeeded in proving

that within the stem the response to auxin differs in adjacent layers of tissue, the outermost having a greater *inherent* ability to extend, under optimal auxin supply, than the inner layers. This differential response does not depend necessarily on morphological differentiation among the cell layers, since it is shown in organs, like the coleoptile, in which practically no morphological differences exist from layer to layer. Such inherent differences must presumably be ascribed to differences in accumulation of the hormone or differences in the supply of other factors needed to give the response.

The more closely it is examined, at least in plants, the more the process of growth seems to be inseparable from all other functions of the cell. In the analysis of auxin action, an increasing importance has come to be attached to the other factors which act together with auxin: these include sugar, inorganic salts, and certain nitrogenous substances, apparently including some of the purines. Growth is also linked in some way to protoplasmic streaming, since auxin acts on both over the same range of concentrations and in a closely-related way. We have found also that growth is linked to a part of the respiration system,—probably a special small part of the total respiration, but the part which is necessary for growth, and in which auxin acts as some kind of coenzyme or catalyst. Thus the study and analysis of growth involves the consideration not only of water uptake and such obvious accessories, but of cellular metabolism as a whole.

PHYSICAL FACTORS OF GROWTH

By

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INTRODUCTION

The broad title of my discussion is "Physical Factors of Growth". I have decided to confine myself within this title primarily to an account of some experiments on factors that determine the axis of differentiation and growth at an early stage in the *Fucus* egg: at a stage when growth and differentiation of the zygote first appear. The effects of a considerable number of physical and chemical factors have been investigated in this one egg, which affords some opportunity to attempt correlations. The factors and relations considered link this problem to many others in the field of development and growth.

The effects on the living protoplasm of physical and of chemical factors become largely indistinguishable if the physiological analysis can be carried far enough. This is true both because any physical change inevitably alters biochemical processes and also because the distinction between physical and chemical largely disappears at the molecular and sub-molecular levels at which we believe the ultimate, even if mostly unidentified, processes of differentiation and growth take place.

Embryologists ordinarily think of development as the resultant of quantitative increases called growth and qualitative changes called differentiation. These two processes may be separated quite completely in some cases, and under certain conditions, but ordinarily in development they proceed together with very definite interrelationship. In the case which I am about to present, differentiation and growth take place simultaneously, if elongation and more rapid cell division are considered to constitute growth. Any region of the egg cytoplasm which is caused to form a rhizoid protuberance differentiates and grows in a special manner. It gives rise to a specialized part, just as does the polar lobe of a Molluscan or Annelid egg, or the antipolar region of the starfish egg which gives rise to the primitive gut. In the starfish egg, as in most animal eggs, regions of the cytoplasm are already differentiated before fertilization. The *Fucus* egg, on the other hand, appears to be relatively undifferentiated until the rhizoid protuberance forms in the latter part of the one-cell stage.

Fucus is a Marine Brown Alga which attaches to fixed rock in the upper part of the tidal zone. Like most animals, it is diploid except for a few cell generations leading up to the formation of gametes. Most species are hermaphroditic, but some are dioecious. The spermatozoa

or antherizoids are motile and fertilization normally takes place in the sea water.

The *Fucus* egg is spherical and the nucleus ordinarily lies in the center. The eggs vary somewhat in size but the diameter (65-90μ, average 75μ, in *F. furcatus*) is similar to that of a number of sea urchin and other marine invertebrate eggs. The eggs contain plastids which give them a brownish-green color and they are photosynthetic. The unfertilized egg is naked, but immediately after fertilization jelly is secreted which gradually hardens to form a rigid, close-fitting, cellulose-like cell wall which adheres firmly to the substrate. After fertilization there is a relatively long delay before morphogenesis begins with the formation of a bulging protuberance on the egg. Protuberances form in a population of eggs during the period 12-24 hours (av. 18) after fertilization when the eggs are reared in the dark at 15°C. The protuberance extends and is cut off at about 25 hours by the first cell division. The resulting two cells are very different in shape, and also in developmental fate. The more rounded cell without the protuberance gives rise only to thallus. The most basal part of the cell containing the protuberance also contributes to the thallus, but the extended protuberance itself gives rise to the rhizoid. When the rhizoid protuberance first appears, the developmental pattern and the polarity are determined. Factors which determine the place of origin of the rhizoid protuberance determine the developmental fate of all parts of the cytoplasm of the egg.

FACTORS DETERMINING THE POINT OF RHIZOID ORIGIN

A single egg which develops alone in the dark, as free as possible from gradients or asymmetry of all known environmental factors, forms a single rhizoid and develops normally. It therefore appears either (1) that there is a predetermined polarity in the egg (perhaps imposed by conditions during oogenesis) which however is extremely labile and readily altered or (2) that polarity is determined after shedding by chance environmental differentials of a minor sort. Knapp (1931) has observed that the entrance point of the sperm determines the point of rhizoid origin in the related *cystosira*, and it is not improbable that this is also the case in *Fucus*. Whatever the cause of the original determination, it is readily superceded by the effects of subsequent gradients of physical and chemical factors.

Visible light:

Rosenvinge (1889) and Kniep (1907) and a number of more recent investigators have found that unilateral illumination by white light causes rhizoids to form on the least illuminated sides of the eggs, i. e. on the sides away from the light. The responsiveness of eggs of *F.*

furcatus is not fully developed until about 7 hours after fertilization at 15°C. (Whitaker and Lawrence, 1936).

Hurd (1920) used Wratten filters to separate the visible spectrum into fairly narrow spans of wave-length (red, yellow, green, blue, violet). Sunlight, and carbon and mercury arc sources were used, and intensity was measured, and made comparable in some experiments. It was found that only the shorter wave-lengths of the visible spectrum were effective, 4000 to 5200 or perhaps somewhat more Å, i. e. violet, blue and probably the shorter green. Longer wave-lengths, 5800-7000 Å, had no effect even at relatively high intensity. The same wave-lengths which determined the point of rhizoid origin also exerted a negative phototropic effect on the growing rhizoid. It should be noted here in passing that the visible wave-lengths which are effective in *Fucus* are in general similar to those which cause phototropism in higher plants in which growth hormone (auxin) is involved. Although the relation of wave-length to photosynthesis has not been determined in the *Fucus* egg the wave-lengths which are most effective in determining the rhizoid origin are probably not the most effective in photosynthesis. Hurd also refers to some evidence that ultra-violet may be effective but investigation of this region of the spectrum does not appear to have been pursued. More extensive recent evidence will be considered below.

Electricity:

Lund (1923) reared *Fucus* eggs in the dark in sea water through which a direct electric current was passed so that there was a potential drop across each egg. The rhizoids formed on the sides of the eggs toward the positive pole. The threshold P. D. was 0.025 volts across an egg, and development was not retarded. The rhizoids thus formed at the sides of the eggs at which negative ions, including those of growth substance (auxin), presumably accumulated.

"Group effect":

It is clearly shown by transplantation experiments on animal embryos, and by earlier work on the development of separated blastomeres, that the type of differentiation of a given cell depends not only on the nature of the cytoplasmic contents of the cell but also upon influences from neighboring cells. A simple example is afforded by the sea urchin embryo in the 2-cell stage. In the normal course each of the adjacent cells gives rise to half a larva, but if the two blastomeres are separated each gives rise instead to a whole larva. The factors involved in the effects of cells upon the differentiation of other cells (in some cases referred to as induction) must of course differ greatly in different organisms and situations. *Fucus* eggs exert mutual effects on the differentiation of neighboring cells which act at a surprising distance (e. g. through

0.3 mm. of sea water), so that the technical difficulties of examining into the factors involved are reduced.

It was observed by Rosenvinge (1889), Kniep (1907), Hurd (1920) and Whitaker (1931) that *Fucus* eggs tend to form rhizoids in the resultant direction of neighboring eggs. Hurd found that the effect was strong enough to overcome the response to light if the eggs were within several egg diameters of each other. Whitaker (1931) found that two eggs alone in a dish in normal sea water did not form rhizoids toward each other, but that the attraction ("group effect") depends on appreciable masses of eggs as an attracting body. The effect was also found to be non-specific, i. e. masses of unfertilized eggs of *F. vesiculosus* exerted a strong group effect on developing eggs of *F. evenescens.*

Since one obvious effect of masses of eggs would be to lower pH in the immediate vicinity of the eggs, due to CO_2 and perhaps other acid metabolites, aggregations of various size were reared in sea water acidified with McIlvaine's buffer or HCl and sodium bicarbonate (Whitaker, 1937a). In medium at pH 6.0 the group effect was greatly strengthened so that five (Fig. 1) or even two eggs alone in a dish

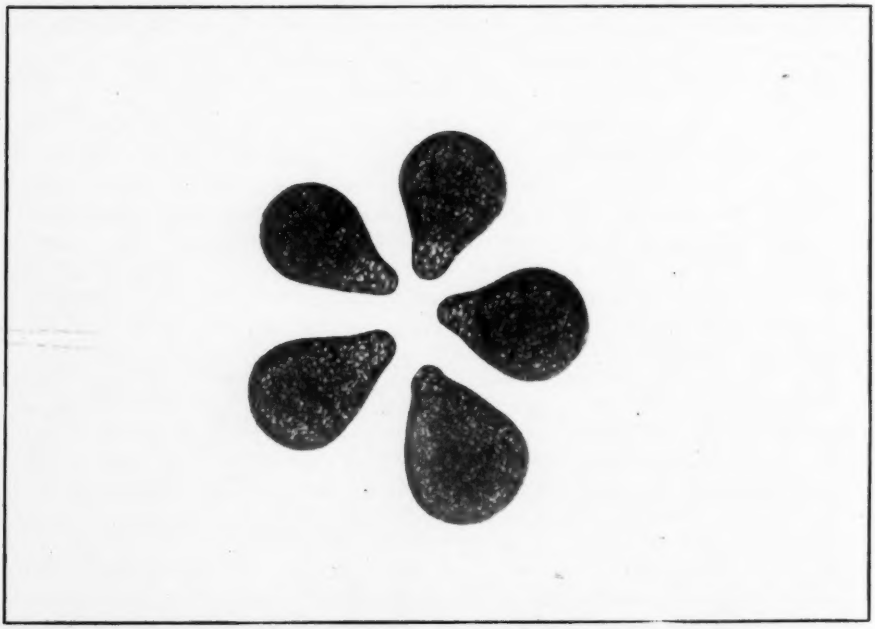

Fig. 1. Photomicrograph of a group of 5 *Fucus* eggs reared in the dark at 15°C in sea water acidified with McIlvaine's buffer to pH 6.0. Rhizoids have formed in resultant directions of neighbors ("group effect").

now formed rhizoids toward each other (90%) at distances up to 4 egg diameters. Even the bottom of the dish, or other surfaces which blocked diffusion of products from the eggs so that they accumulated and formed a gradient now served to attract the rhizoid formation. Either the egg becomes more sensitive to certain gradients when it is acidified, or possibly acidification of the outer surface of the cell membrane permits certain substances (e. g., auxin) to diffuse out more readily and thus actually produce stronger concentration gradients in the medium. The dissociation of weak acids is decreased by acidity and cell membranes are more permeable to molecules than to ions.

Diffusion Gradients:

Single eggs were reared near one end in close fitting capillary tubes so that products from the eggs could diffuse much more freely from one side of the egg (Whitaker, 1937b). Each egg thus developed in a diffusion gradient of its own products (as well as of entering O_2). The effect of pH of the medium on the result was very marked. If the medium was initially at pH 8.0 or below the rhizoid formed in the tube, i. e. on the side subjected to the greatest concentration of substances diffusing from the egg. The effect was stronger with lower pH , as in the case of the group effect, and it appears very probable that the same factors are involved in both cases. The egg responds to diffusion gradients from itself as well as to the products of another egg. It does not appear possible that mitogenetic radiation, as ordinarily conceived, could be involved in this action of a single cell upon itself. When the pH of the medium was above 8.0 the rhizoid tended to form on the opposite side of the egg, i. e. on the side toward the near end of the tube where the concentration of substances diffusing from the egg is at a minimum. This suggests that a negative "group effect" or repulsion of rhizoid formation might be expected between two eggs alone in a dish in alkalinized sea water if it is true that the same factors are involved in the tube experiments and in the group effect. Experiments were carried out to test this point.

Negative "group effect":

Whitaker and Lowrance (1940) tested the effect of alkalinized as well as acidified sea water upon the intensity of the group effect between two eggs alone in a dish. It was found that in more alkaline sea water there is in fact a tendency for rhizoids to form on the sides of the eggs away from the neighbor, i. e. there is a negative group effect. This is in agreement with the results from diffusion gradients in tubes. The actual pH of the medium at which reversal takes place is higher in the case of the tubes (pH 8.0) than in the case of two neighbors in a dish (pH 7.6) as might be expected since the greater impediment to diffusion in the tubes undoubtedly lowers pH in the immediate vicinity of the eggs. This negative group effect is at present difficult to recon-

cile with a general explanation based either on hydrogen ions or CO_2 or on auxin (see below).

pH gradients:

It has been shown that acidity intensifies the group effect. A generalized acidification of the medium does not itself provide any asymmetry of environment, however, and gradients must be looked for in the operation of the group effect. Since products from the eggs diffuse away in more or less radial fashion, they will be most concentrated on the sides of eggs toward neighbors where the concentrations from different eggs are additive. Eggs in the dark produce CO_2 and perhaps other acid metabolites so that pH gradients may be expected to result with the greatest acidity on the side toward the neighbor. To directly test the effect of pH gradients, individual eggs were reared between the tips of two diffusion pipettes at different pH (Whitaker, 1938). One of the pipettes contained normal sea water, like the medium, and the other contained sea water acidified to pH 6.0 with McIlvaine's buffer or HCl and sodium bicarbonate. Under these conditions the rhizoid formed on the most acid side of the egg (Fig. 2)

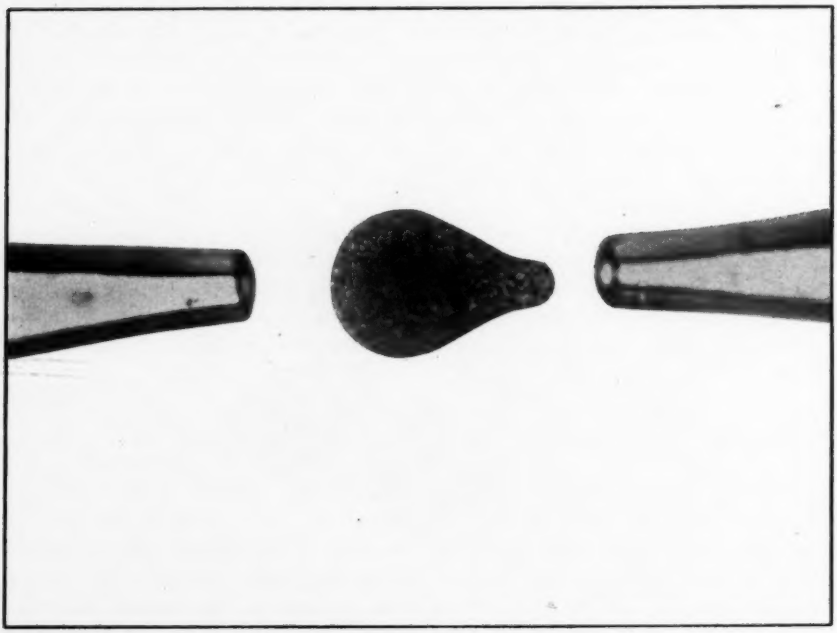

Fig. 2. Photomicrograph of typical *Fucus* egg which has developed in sea water in a pH gradient. The pipette on the left hand side contains sea water at pH 8.0, while the pipette on the right hand side, toward which the rhizoid has formed, contains sea water acidified with McIlvaine's buffer to approximately pH 6.0.

even if the pipettes were withdrawn just before the rhizoid protuberance began to form. If the acid pipette was too acid (pH 5.6) the rhizoid formed instead on the other side of the egg. It might be supposed that this is due to injury, or the impossibility of rhizoid formation at such extreme acidity, but this is not the case since normal rhizoids form, although somewhat delayed, in medium buffered at pH 5.5. It appears that the gradient is as important as the absolute pH. Whether or not a rhizoid forms at a given region of the egg surface depends as much on the pH at other regions of the egg surface as on the pH at the region in question (see Child, 1940).

The sea water was well aerated after acidification to remove the excess CO_2 produced by the action of acid on carbonate in the sea water, but in a system containing carbonate more CO_2 must have been produced when the acidified sea water diffused into contact with less acid sea water. The pH gradient in the sea water therefore inevitably produced a parallel CO_2 gradient, and the experiments do not distinguish between the effects of hydrogen ions and of CO_2. It can be concluded however that the pH -CO_2 gradient determines the developmental axis, and provides an adequate although not exclusive explanation of the group effect.

Auxin:

The response of the *Fucus* egg to light suggests that auxin or an auxin-like substance may be involved in the rhizoid forming process. This is further suggested by the softening of the cell wall that takes place where the rhizoid forms and by the response of the eggs to acid. The auxins are weak acids and there is evidence that the undissociated molecule is most effective physiologically since hydrogen ions increase auxin activity (Bonner, 1934; Thimann, 1935; Went, 1935). It is possible that this relates as much to penetrability as to actual activity. Thimann and Went and others (see Thimann, 1935) have further shown that auxin induces root differentiation. While the rhizoid is not a root it in some ways resembles a root. DuBuy and Olson (1937) succeeded in extracting auxin from *Fucus* eggs, without determining which of the auxins it is, and Olsen and DuBuy (1937) found that rhizoids form on the side of greatest concentration of beta-indole acetic acid (hetero-auxin), or its potassium salt, when these substances are applied in high concentrations to one side of the egg. Van Overbeek (1940) has recently determined that auxin is present in the Brown Alga *Macrocystis* in concentration comparable to that of auxin-a and auxin-b in higher plants, but it is beta-indole acetic acid or a similar substance which is present rather than auxin-a or auxin-b. Its distribution in *Macrocystis* supports the view that it is a functional hormone. However, beta-indole acetic acid is apparently produced as an unused waste product by certain moulds, and by animal tissues as well, e. g.

urine and blood of mammals; animal embryos (Robinson and Woodside, 1935; Rose and Berrier, 1935). Its presence in the *Fucus* egg therefore does not conclusively prove its normal function. The strong effect of acid on the *Fucus* egg may well be due in part to its activating effect on auxin, but it appears highly probable that so active an agent as the hydrogen ion would also act on other steps in the rhizoid forming process as well. Further evidence relating to hetero-auxin is given below under centrifuging.

Temperature Gradients:

Lowrance (1937) reared *Fucus* eggs in steep temperature gradients. The rhizoids formed on the warm sides of the eggs provided the temperature drop across an egg was more than 0.4°C. The temperature gradient is effective even if it is withdrawn some hours before the rhizoid protuberance begins to form. Respiration and other vital activities must proceed more rapidly on the warmer side of the egg. Among other things, a pH gradient might be expected to result since with rise in temperature CO_2 production would increase more than rate of diffusion away.

Mechanical elongation:

When cells are elongated, the division spindle commonly lies in the long axis of the cell so that the plane of cell division is determined. This does not usually affect the morphogenesis in animal eggs, although Lindahl (1936) has found that when sea urchin eggs are stretched by passing through capillaries the first end to emerge tends to become ventral so that in this case the dorso-ventral axis is determined. When *Fucus* eggs are elongated by being sucked into a pipette of small diameter they retain the elongated shape after being blown out if they are taken into the pipette early in the period when the cell wall is hardening. This is due to hardening of the cell wall in the elongated shape. When elongated eggs are reared in normal sea water at pH 8.0; the rhizoid forms at or near one end of the long axis so that the primary polarity, as well as the plane of cell division, is determined (Whitaker, 1940a). There was not much correlation between rhizoid formation and the end of the egg which left the pipette first or last. Elongated eggs lie with the long axis parallel to the bottom of the dish, and when they are reared in medium at pH 6.0 the increased responsiveness of the egg to gradients caused by this impediment to diffusion overcomes the shape effect so that the rhizoid forms toward the bottom of the dish, at an end of a short axis.

The relation of shape of the cell wall to the direction of growth at pH 8.0 bears some resemblance on a relatively gross scale to the growth of chick mesenchyme cells *in vitro* in Weiss' experiments (see Weiss, 1939). When the medium is subjected to stresses which align its colloidal components the mesenchyme cells send out processes in accord-

ance with the orientation of the medium. In *Fucus* the direction of stress of the cell wall appears to guide the direction of growth of the cell within. At present it is not clear what relation the determination by shape bears to other determining factors.

Centrifuging:

The protoplasmic inclusions of many animal eggs have been stratified by centrifuging. The development may be modified, but the primary polarity has not been determined by the pattern of distribution of the inclusions (Morgan and Spooner, 1909; Lillie, 1909; Conklin, 1931; Taylor, 1931; Morgan and Tyler, 1935; Beams and King, 1938). Twinning has been induced (Tyler, 1930; Harvey, 1935), and King and Beams (1938) have altered the diminution phenomenon in *Ascaris* by ultra-centrifuging. Centrifuging has been found to determine the dorso-ventral axis in sea urchin eggs (Runnström, 1926; Lindahl, 1936) and in the spirally cleaving *Urechis* egg (Pease, 1938).

Knapp (1931) found that centrifuging caused the eggs of *Cystosira* to form rhizoids at or near the centrifugal pole. Schechter (1934) observed that in an electric current the Red Alga *Griffithsia* forms rhizoids toward the positive pole, as does *Fucus* (see above), and also that chromatophores migrate toward the positive pole. He then centrifuged the chromatophores to the centrifugal end to see if they were the causal agent, and found that not rhizoids but shoots formed centrifugally (Schechter, 1935). Centrifuging thus had an effect on the polarity.

Fertilized *Fucus* eggs were embedded at random in sea water-agar and stratified in the centrifuge at 3000 $\times$ g. (Whitaker, 1937). The embedding prevented orientation of the eggs on the centrifuge. Most eggs remain stratified until after the rhizoid formation, and when the eggs develop in normal sea water at pH 8.0 the rhizoids form at the centrifugal pole (Fig. 3). Eggs in which the visible protoplasmic inclusions redistribute, however, form rhizoids at random with respect to the earlier stratification, i. e. the effect of centrifuging is lost if the inclusions redistribute. Essentially the same results are obtained by ultra-centrifuging at 50,000 to 200,000 $\times$ g. at any time nearly until the rhizoids actually begin to form (Whitaker, 1940b). When ultra-centrifuged eggs are reared instead in sea water acidified to pH 6.0 with McIlvaine's or Sorensen's buffer, the development is strikingly reversed with respect to the stratified zones, i. e. the rhizoids form centripetally. A tentative explanation of this reversal might be that the centripetal oil cap end has less buffer capacity (and possibly also a thinned membrane) so that its pH is more affected by the pH of the medium. If so, the centripetal pole would be the basic end of an internal pH gradient when the egg is in normal sea water, but it would be the acid end in medium which is more acid than the protoplasm at

large. If the pH of the medium were the same as the average pH of the protoplasm, no internal pH gradient would result from this effect, and it is actually observed that the rhizoids form at random with respect to the strata when the medium is at pH 6.3. Other interpretations are of course possible. An amphoteric phenomenon may be involved, or a change in relative fat-water soluability of some essential substance with changed pH.

It has already been pointed out that one mode of action of hydrogen ions on *Fucus* eggs might be through increasing the concentration of non-dissociated, molecular auxin. Another way to increase the con-

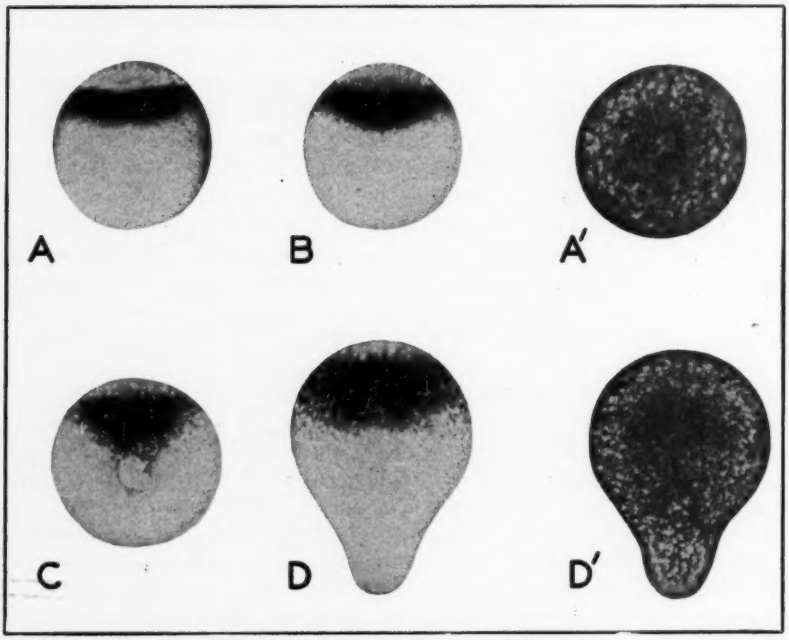

Fig. 3. Photomicrographs of typical developing centrifuged and normal *Fucus* eggs. (A) shows an egg 23 minutes after being centrifuged for 20 minutes at 3,000 × g. The centrifuging began shortly after fertilization, and the centrifugal end is below. Chloroplasts are concentrated in the dark centripetal band. (B) shows the same egg 15 minutes later, and already inclusions in the dark band are seen to be diffusing back to some extent. (C) shows another egg from the same sample 3 hours and 10 minutes later. The nucleus has emerged from the dark stratum within which it was earlier concealed. (D) shows another egg from the same sample 13 hours and 25 minutes later. It may be seen, especially by referring to the remnant of the centripetal cap, that the rhizoid protuberance has formed at the centrifugal end of the egg. (A′) and (D′) are normal non-centrifuged eggs in stages corresponding to (A) and (D) respectively. All eggs were reared in the dark at 15°C. at pH 7.9-8.0.

centration of molecular auxin is to add auxin while maintaining constant pH. Since the pH of the medium determines the percentage of stratified eggs which form rhizoids on the centrifugal and centripetal halves, the effect of hetero-auxin (beta-indole acetic acid) was also tried at pH 8.0, and also at pH 6.3 which should be more favorable for hetero-auxin penetration (see Albaum, Kaiser, and Nestler, 1937). In both cases the hetero-auxin, like acid, favored centripetal rhizoid formation. Its effect was not very pronounced, however, and

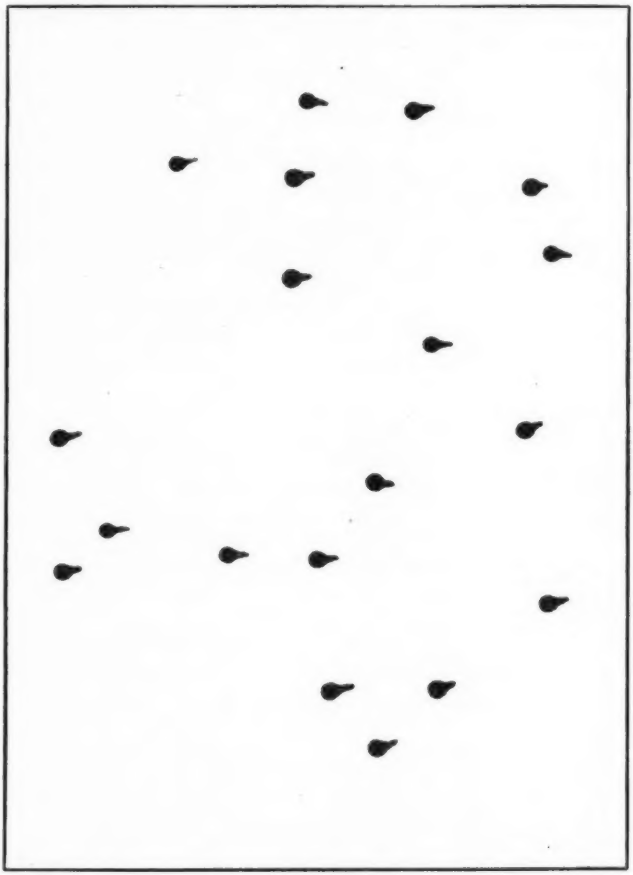

Fig. 4. Photomicrograph of a small area of the bottom of a quartz culture vessel in which *Fucus* eggs have developed. These eggs were irradiated from the left hand side with monochromatic ultra-violet light ($\lambda = 2804$). Approximately 10,000 eggs per mm^2 was applied during the period 7-9 hours after fertilization (15°C.), and the rhizoids later developed on the sides of the eggs away from the source of radiation.

as the concentration was increased it became toxic before becoming nearly so effective as acid. It appears improbable, therefore, that acid is acting in this case merely through its effect on the dissociation or activity of hetero-auxin.

Ultra-violet irradiation:

When *Fucus* eggs are irradiated from one side with adequate dosages of ultra-violet light, 97-100% form rhizoids on the sides away from the source of radiation (Fig. 4) (Whitaker, 1941). The responsiveness of the eggs to the wave-length 2804 Å. increases gradually after fertilization and reaches a maximum at about 7 hours after fertilization at 15°C. The earliest rhizoids begin to form in a population at about 12 hours after fertilization. The low responsiveness of eggs which are irradiated soon after fertilization may perhaps be due to recovery before the rhizoids begin to form.

The response of a population of eggs to λ 2804 Å is proportional over a wide range to the logarithm of the total dosage applied. A sufficiently strong dosage inhibits rhizoid formation altogether without causing cytolysis.

There are of course many substances that might be altered or destroyed by λ 2804 Å., but two in particular suggest themselves in the present case. Proteins are denatured (first order reaction) by ultra-violet of wave-lengths less than 3000 Å (Anson, 1938) and denaturation involves pH. changes (Clark, 1936). Auxin is inactivated by ultra-violet (Went and Thimann, 1937) and presumably hetero-auxin is also inactivated.

Longer wave-lengths (3130 and 3660 Å.) also direct the *Fucus* eggs but they are very much less effective, while shorter wave-lengths (2654 to 2345 Å.) are highly effective. It remains to be seen whether the effectiveness of wave-lengths shorter than 3000 Å is proportional to protein absorption, and whether the addition of hetero-auxin will cause rhizoid formation in eggs which have been inhibited by over-doses of destructive ultra-violet.

SUMMARY

The *Fucus* egg is relatively undifferentiated until the rhizoid protuberance forms in the latter part of the one-cell stage. Gradients of a number of physical and chemical factors, the presence of neighboring egg cells, and internal segregation of the protoplasmic inclusions by centrifuging, may determine the axis of differentiation and growth. These factors determine the developmental fate of all parts of the egg protoplasm.

The effects of some of these factors can be resolved to a common action of hydrogen ions and CO_2. It is probable that auxin or an auxin-like substance may be involved in a step in the rhizoid forming process.

but if so, effective factors undoubtedly also act on other steps in the rhizoid forming process as well. The initiation of the axis of morphogenesis in the *Fucus* egg by externally applied gradients of physical and chemical factors is in agreement with Child's (1940) physiological gradient generalization. If the morphogenesis is to be fully understood, however, it is also necessary ultimately to know the specific nature of the substances and reactions involved in the actual differentiation and growth.

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DISCUSSION REMARKS

To D. M. Whitaker's Paper On "Physical Factors Of Growth"

By

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There are a number of points which occurred to me as I listened to Dr. Whitaker's beautiful experimental analysis of the control of polarity in the *Fucus* egg. These might serve as questions and subjects for general discussion.

(1) The *Fucus* egg appears to be a cell which is completely symmetrical and consequently apolar. The nucleus is in the center of the egg and all axes drawn thru the center are alike. In this respect it differs from such an egg as that of the sea urchin, Arbacia, where the nucleus of the unfertilized egg is excentric and an axis can be drawn in one direction. We do not yet know whether in the sea urchin egg the excentricity of the nucleus is connected with the original position of the oocytes in the germinal epithelium or to the position of polar body formation or to the action of gravity, since the nucleus is lighter than the cell fluid and would slowly float upward in an undisturbed cell.

(2) In most eggs it is difficult to change the polarity. The *Fucus* egg differs in that a polarity can be easily impressed on it. In addition, the egg is large enough to experimentally subject it to gradients. It is extraordinarily favorable material and the question arises whether other algal or fungal cells show a similar response to the same conditions which control polarity in *Fucus*. One similar case I recall was described by Beams (1937) who noted that 80% of yeast cells in a gravitational field of 200,000 $\times$ g, bud parallel to the direction of the field.

(3) Especially noteworthy is the response of the *Fucus* egg to such a wide variety of gradients—light (both visible and ultra-violet), temperature, electrical potential, a centrifugal field, auxin, H-ion concentration and group influence of adjacent cells. It would be interesting to know whether oxygen, CO_2, osmotic pressure, salt (Ca, Mg, Na, or K) and magnetic field gradients likewise influence the appearance of rhizoids. Also whether in complete darkness rhizoid formation is possible in a completely uniform field, which might be obtained by gentle agitation of parthenogenetically activated *Fucus* eggs.

(4) Since the pH has such an important effect on rhizoid formation, it should be determined whether the necessary pH gradient is intracellular or extracellular. This could be done by using penetrating

acid and alkalies like acetic acid and ammonia contrasted with non-penetrating acids and alkalies like HCl and NaOH, to produce the gradient. Of course bicarbonate free sea water must be used as a basic medium to prevent complications due to liberation of CO_2 on adding acid. If rhizoid formation is due to intracellularly produced auxin, whose action depends on its dissociation, the pH gradient would of necessity have to be maintained by acids or alkalies capable of penetrating the *Fucus* eggs. However, it must be borne in mind that the activity of auxin at different pH values in higher plants may be connected with the penetration of auxin into a cell when present as free acid, and non-penetrations when present as salt.

(5) It would be interesting to know if there is any specificity in group effects, i. e., could a clump of yeast cells have the same effect on rhizoid formation as a clump of *Fucus* cells.

(6) Finally, the mechanism of rhizoid formation must be thoroughly understood to explain why so many diverse gradients influence the polarity. This involves a study of differential effects within a cell, particularly the differential distribution of enzymes within the cell. It is to be presumed that a cellulase must accumulate on the side of the egg cell where the rhizoid is to form. By digestion and thinning of the cellulose membrane a bulge would form which is to be the rhizoid. Must we believe that centrifugal force throws zymogen granules to this region or that a certain pH is necessary for the action of the cellulase? The answer cannot be quite as simple as this but the whole question of the distribution and activation of enzymes within cells must be involved. I would suggest that studies on *Fucus* eggs similar to those of Holter (1936), Shapiro (1935), and Ballentine (1940) on sea urchin half eggs might be used to determine the distribution of intracellular enzymes. If the *Fucus* egg can be pulled into two halves by centrifugal force in an early stage of development, before the cellulose membrane has hardened, a new series of experiments on the distribution of enzymes and of possible rhizoid forming material could be carried out.

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CELL DIVISION IN RELATION TO DIFFERENTIATION

By

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This discussion will be limited to the problem of the relation of cell-division to differentiation—i. e. cellular differentiation, histogenesis or cytogenesis. In any discussion of this kind it is always necessary to clarify the topic by attempting to define the terms included in it. The term "cell" and the implications of the concept of the basic organization of living things have been recently discussed at length by several groups on the occasion of Centennial Celebrations of the enunciation of the so-called Cell Theory with which the names of Schleiden and Schwann have been so closely linked. It seems unnecessary at this time to recapitulate the arguments for and against the individuality of the cell—the concept is a useful one even though in many circumstances its inadequacy is disturbing and we often wish that living organisms did not present so many apparent departures from what would seem to be a simple unit plan of organization.

Before beginning work on this topic it seemed clear that *cell division*, the means by which these microscopic units of structure were multiplied, could be described in terms of two sharply contrasting methods—mitosis and amitosis. Further study, however, soon revealed that there are all degrees of expression of the mitotic phenomenon and that there are situations in which mitosis is so modified, or certain phases of the phenomenon so greatly suppressed, that it can be distinguished only with difficulty from amitosis, if indeed amitosis is a fundamentally different method of cell multiplication. I shall return to this point later and attempt to illustrate some of the complications encountered when one attempts to separate mitotic from amitotic phenomena.

Bloom (1937) has defined cellular differentiation "as the process which results in specialization of a cell as measured by its distinctive actual and potential functions". In this definition Bloom does not mean to exclude morphology entirely but regards morphology and function as two aspects of one quality. "The morphology of a cell may be considered to be a reflection of its actual functions" (p. 592). The duality of this definition is further emphasized by the author when he states "the ideas of the distinctiveness of cellular functions and potencies must be included in this definition if we are to ascertain how cells differ from one another". Weiss (1939) also writes that "the state of cellular differentiation must be conceived of in terms of attending *potentialities* and *activities*, rather than by reference to definite mor-

phological structures" (p. 96). How much more simple the situation would be for the histologist untutored in experimental embryology if cellular differentiation could be defined as the attainment of a specific functional capacity, with the morphological expression attendant upon this capacity, without being continuously concerned with the potencies or possibilities latent in a cell which may or may not be realized at some future time under different environmental conditions! However, in accordance with the duality of our definition there must be double criteria of cellular differentiation in terms of both actual and potential functions. Therefore, a cell must be judged not entirely on its present state but also in terms of one or more possible future states.

Moreover, cellular differentiation must be further distinguished from cellular changes, sometimes striking, that reflect temporarily the reactions of cells to new environmental conditions or the withdrawal of existing stimuli, without resulting in any change in potential functions. To such a situation in which these cellular changes are reversible without residue, Weiss (1939) applies the term "modulation".

The verbal picture at least, if not also our mental processes, is further clouded by the term "dedifferentiation". Unfortunately this term has been subjected to loose usage if not outright abuse. The concept of "cellular dedifferentiation" is in need of exact definition. Obviously many persons do not use the term in the sense of Champy's "dedifferentiation" by which it is implied that all types of cells become reduced to a single indifferent cell type, eventually comparable to cells of the early embryonic stages.

The terms "tissue dedifferentiation" and "cellular dedifferentiation" have come to denote rather a manifestation of the powers of adaption of cells to an abnormal environment, such as in tissue culture, which is never or very exceptionally found in the living organism. If such cells can redifferentiate under more favorable cultural conditions to reacquire their specific functional capacities, then the "dedifferentiation" and subesquent "redifferentiation" would seem to lie within the area delimited by the term modulation. When under these conditions the cell remains unable to "redifferentiate", Kasahara (1935a, b) feels that this should be spoken of as "deflection", the cell being turned away from the line of ontogenesis.

The problem has been stated in other terms by Bloom (1937). "If modulation is the loss of some actual functions without a change of potencies, then 'dedifferentiation' would be the loss of some actual functions and an increase in potential functions. 'Redifferentiation' would be the return to a position of greater specialization in actual and potential functions", p. 592.

This implies that cellular dedifferentiation, in the original sense, is an actual phenomenon and that such cells do reacquire potencies temporarily lost. On this point the evidence is certainly not all in. Conversely it may eventually be demonstrated that what appear to be true differentiations may only be modulations, that what appears to be the final level of specialization is temporary rather than permanent, reversible rather than irreversible. Even the neurone of the adult mammal may conceivably be demonstrated by new techniques to have other possible potencies of development. The evidence from experimental embryology and tissue culture forces us to conclude that so-called cellular differentiation, dedifferentiation and redifferentiation can not be measured alone on the basis of present structure and function and that at each level we must further test for the existence of other potencies of development.

Another source of confusion of terminology results from speaking of cells as partially or incompletely differentiated. This difficulty, as I pointed out earlier, is partly inherent in the dual nature of the definition of cellular differentiation. For example, erythroblasts may be spoken of as partially differentiated erythrocytes even though we at present believe that this cell line is finally restricted and will give rise to nothing else but mature erythrocytes. On the other hand, a basal cell of the epidermis of an amphibian is also regarded as partially differentiated. In the latter instance this conclusion is not based on the actual functional and structural characteristics of the cell in question but is due to the fact that we realize this cell may give rise to components of mucous glands or granular glands or even lateral-line organs. In the first example, the cell is fully restricted, that is differentiated, and proceeds to a determined end or final functional specialization. In the second, differentiation may be divergent as the potencies of this cell are not finally restricted.

In casting about for a term to cover the first situation I have benefited from the friendly counsel of Professor Hoadley who suggested *histoteleosis and cytoteleosis* as proper designations for the process by which a cell-line or an individual cell, already irreversibly differentiated from the developmental point of view, proceeds to final specialization. Accordingly we can speak of a cell in the erythroblast-line as a cell undergoing early histoteleosis and the erythroblast which transforms without further cell-division directly to an erythrocyte, as being involved in a late state of histo- or cytoteleosis. Both terms imply that the cells concerned are proceeding to a complete, that is, final and irreversible specialization. I have been unable to find or coin comparable terms to designate a cell line or cell which is partially differentiated in the sense that its potencies have not been finally restricted, or which remains partially in "open term".

After this long and tedious digression, we may return momentarily at least to the topic I announced for discussion: "cell-division in relation to cellular differentiation". As Bloom (1937) points out, "cellular multiplication and cellular differentiation are distinct processes, each of which usually takes place only in the absence of the other" (p. 611). This same idea is reiterated by Weiss (1939) in many places in his stimulating book on Principles of Development. Quoting: "In general it can be said that, the more specialized a cell is in its structure and function, the less apt it is to divide. Mitotic ability seems thus to be limited more by the degree than by the type of specialization" (p. 59). "The act of mitosis causes such a stirring up of the cell content that many of the internal features which the mother cell has managed to produce during its previous inter-kinetic phase are wiped out or at least upset." . . . "But while the mitotic upset has far reaching consequences for the physical structures of the mother cell, it does not interfere very seriously with the chemical differentiations a cell might have acquired" (p. 76). "Cellular differentiation and cellular multiplication are two processes which, if not strictly mutually exclusive, are nevertheless markedly antagonistic in their tendencies" (p. 85). "A differentiating cell, therefore, gradually loses its capacity to divide and factors promoting differentiation automatically reduce proliferation" (p. 424).

Although the authors just cited have the problem of the relationship between cellular differentiation and cell division clearly in mind, unfortunate generalizations have found their way into the literature and the apparent inability of certain cells to divide has been accepted as primary evidence of their high degree of specialization.

This morning I propose to question the generalization of the existence of a fundamental antagonism between division and differentiation, an antagonism that derives primarily from cellular differentiation per se: that is, to determine whether the process of differentiation causes a cell to lose this capacity, or whether one must seek other causes of the disappearance of this capacity.

It has long been apparent that factors favorable to differentiation are generally unfavorable to cell proliferation. Accordingly, we must determine whether the incapacity to divide is due to intrinsic or extrinsic factors, or an interaction between them. May not the capacity remain unexpressed under an unfavorable environment to find expression when the environmental conditions are subsequently modified? At the outset it must be admitted that certain physical aspects of cellular differentiation practically preclude further typical cell division, but even in these cases we sometimes find a residue of expression of the mitotic phenomenon which is usually centered about the nucleus.

Apparently the factors unfavorable to cell division may press more heavily on the cytoplasm than on the nucleoplasm.

Furthermore, the eventual and obvious loss of mitotic activity which does occur in the life of a cell may not be the direct result of cell-differentiation but may be an outward expression of a process of aging—a process much less obvious than differentiation but one which progresses inexorably to terminate in senility and death. That cells in the course of ontogeny lose the plasticity and adaptability of young cells is amply demonstrated by the small measure of success attending the culture of adult tissues *in vitro*. It is especially unfortunate that in most cases we lack morphological criteria to determine the rate or the degree of the aging process. We might also consider how long this insidious process of aging may be operative before the evocation of a mitotic response becomes impossible. Is rejuvenation possible?

If this viewpoint is tenable, we may assert temporarily, or at least until the discussion begins, that differentiation is not related causally to the inhibition of cell division but that the loss of mitotic activity is the last restriction laid upon a cell, i. e., it must proceed to its determined destiny as a single unit rather than to have portions of its substance distributed to other similar units. Of course the counter viewpoint may be entertained that the processes of differentiation and aging cannot always be separated, except as an abstraction, since they may have a common temporal relation to the life of the cell.

In order to limit further the topic under discussion, I propose to consider cell-division only in relation to those cells which are generally conceded to have entered upon the process which has been designated histo- or cytoteleosis. The restriction does not unduly favor my general thesis since cells which are only partially restricted in their potencies usually give evidence of considerable mitotic capacity.

Cell division carries the implication that there are always individual, discrete units which may be multiplied by this process. However, cell individuality cannot always be assessed by the criterion of morphological delimitation. Morphological discreteness is not constantly encountered. As Weiss (1940) aptly states: "The only definitely discrete element in a cell is the nucleus and since each nucleus keeps protoplasm within a certain radius under its control, protoplasmic territories have the value of cells, no matter whether their boundaries are marked by visible surfaces or merely by a change of physiological properties along the border" (p. 99). Accordingly, any discussion of cell-division cannot entirely ignore these limitations of the concept of cell individuality.

Typical mitosis can no longer be regarded as a unitary phenomenon. It is in reality a combination of several component processes involving

several different constituent parts of the cell. Ordinarily these processes are perfectly integrated and the final result is accomplished by a precise coordination of activities that occur at the proper place and at the proper time. In its fullest expression, asters, centrosomes, centrioles, spindle, nucleus, chromosomes, Golgi apparatus, mitochondria, etc. follow a definite pattern of behavior and as the culminating event the cell is separated into two daughter individuals. However, not all the cellular components may always be visibly present to participate in these reactions and some of the reactions that usually occur may not proceed to completion or may be entirely omitted. In other words, processes which ordinarily are integrated may be dissociated from one another and in some instances do not seem to be greatly impaired by this dissociation. This situation is reflected by the appearance in the literature of such terms as monocentric mitosis, modified mitosis, abortive mitosis, endomitosis, pyknomitosis and pseudoamitosis.

If, as it appears, the mitotic processes can be dissociated it remains to inquire as to which process is the essential one, if it be possible to make such a determination. This point may be clarified, perhaps partially answered, by citing some selected examples.

During the development of the megakaryocyte of the mammalian marrow, the nucleus first hypertrophies and becomes lobulated. This is followed by series of highly modified mitoses primarily involving the nucleus. The centrioles divide several times and a complicated multipolar metaphase appears. There is no true telophase with the formation of daughter nuclei which subsequently fuse to form the complex nucleus. Instead, the nuclear membrane forms about the irregular chromatin mass. This process may be repeated an unknown number of times producing a high degree of polyploidy (Porter and Ward, 1940). These nuclear phenomena are not followed by cytoplasmic cleavage, but the cell continues to increase in size.

In the nurse cells of the ovary of Drosophila, Painter and Reindorp (1938) have described an interesting case of the development of polyploidy in cells in which the nuclear membrane is persistent. The spindle mechanism is absent and the reduplicated chromosomes emerge as in early prophase, but the homologous elements are not separated. The indirect evidence based on the discontinuous increases in nuclear size lead them to estimate that eight such division cycles may occur resulting in nuclei which are at least 512-ploid. This and other considerations caused Painter (1940) to conclude that such contributions of nuclear material from nurse cells to the ovum may explain the rapid synthesis of chromatin which occurs during the rapid cleavages of early development. Painter suggests that a similar phenomenon may occur even in the germinal vesicles of ova which lack nurse cells. No detailed study has as yet been made to determine whether such intra-

nuclear division cycles occur, but he points out that the amount of chromatin involved in polar body formation is disproportionately small considering the total volume of the germinal vesicle. This increase in size is well illustrated by the figures of Evans and Swezy (1931), of the nucleus of the growing ovum of the dog.

Still more striking is the condition described by Berger (1938) in the intestinal cells of the larval mosquito. These cells increase greatly in size without division but at pupal metamorphosis the dividing cells may contain chromosomal complexes of 96, 48, 24 and 12. The diploid number is 6. The multiple chromosomal complexes apparently arose by 3 or 4 successive multiplications while the nucleus remained in the typical diffuse or "resting" condition. The chromosomes are later reduced to the diploid number by a series of somatic reductions. This extreme case of endomitosis furnishes evidence of chromosomal multiplication without outright mitotic activity, or even any apparent change in the "resting" nucleus.

It is perhaps opportune to refer at this time to the classical case of the cells of the mammalian liver, especially of rodents, in which cell size, nuclear size and nuclear number may vary greatly (Figs. 1 to 8). The hepatic cells, so far as we know, have reached their final functional differentiation (histoteleosis) and yet the persisting capacity of these cells to multiply by mitosis, and perhaps by amitosis, has been demonstrated under a great variety of experimental conditions (Jacobj, 1925 to Pfuhl, 1938). Whether these nuclear variations in size and number are due to dissociation of certain of the mitotic processes, to true amitosis or some other phenomena has not been satisfactorily determined but the use of colchicine should aid in solving this puzzle.

A still different situation is encountered in the granular glands of the amphibian skin. Here, as secretion granules accumulate, the cell boundaries soon disappear and the nuclei are found scattered about the periphery of the gland. In Triturus, simple giant nuclei and lobulated nuclear complexes are frequently seen (Dawson, 1937). In Necturus, occasional greatly elongated nuclei are found (Dawson, 1920), while in Rana pipiens, rows of nuclei of normal size, frequently arranged in striking geometric patterns, have been described (Vogel, 1940). These "naked" nuclei are occasionally seen in mitosis but the history of the nuclear changes has not been carefully followed and it is not known whether the giant forms are the results of nuclear endomitosis, fusion following mitosis or amitosis, or simple hypertrophy. Similarly we are quite in the dark as to the origin of the nuclear rows.

Having considered some of the many departures from the normal sequence of events in mitosis, it may be well to attempt an evaluation of the situation in vertebrate skeletal muscle. Here the obvious mitotic

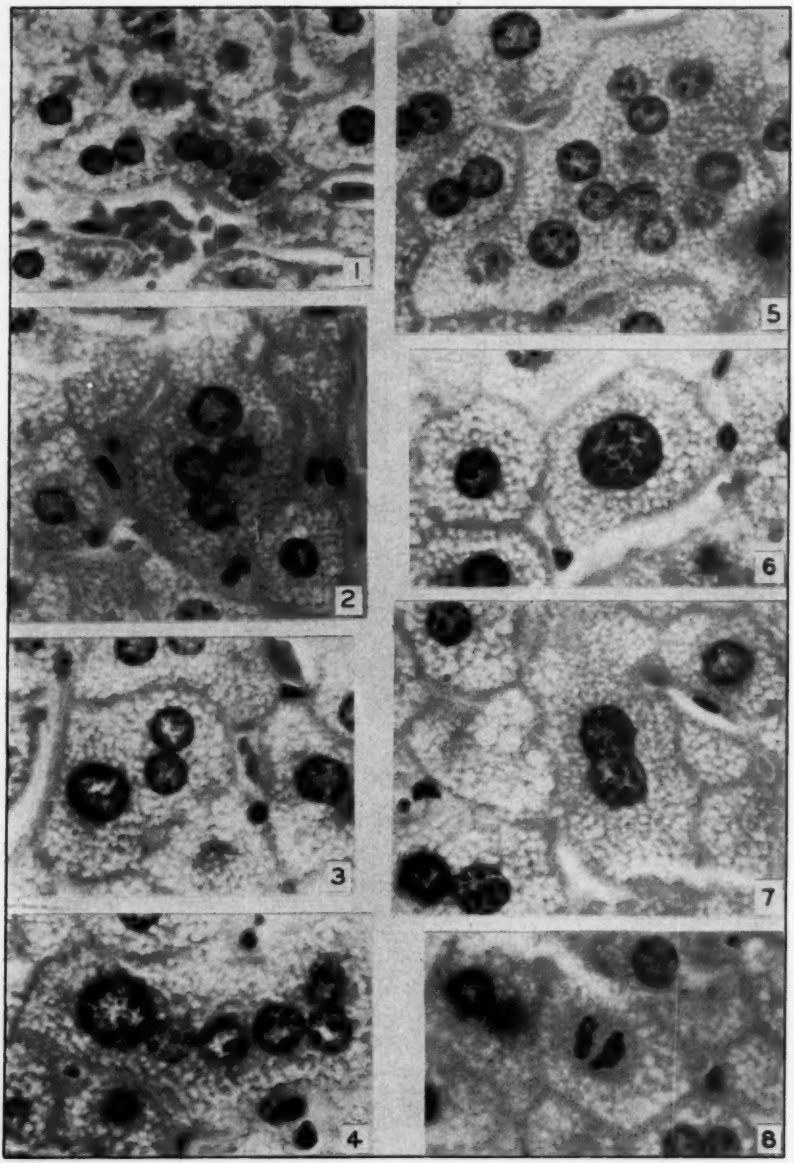

PLATE I

Sections of the liver of an apparently normal, adult rat. The tissue was fixed in Helly's fluid and stained with Ehrlich's haematoxylin and eosin. All figures are photomicrographs taken at a uniform magnification. The variations in cell size, and nuclear size and number are clearly shown. Since the sections were not serial, the total number of nuclei in the individual cells could not always be determined.

EXPLANATION OF FIGURES

1. Three binucleated liver cells showing the normal size of the nuclei.
2. A large hepatic cell with four larger nuclei.
3. A cell with three nuclei.
4. A cell with eight nuclei (one large and six smaller). The two nuclei at the right are almost out of focus.
5. A large cell with at least twelve nuclei. Several are not in clear focus.
6. A large cell with a single large nucleus.
7. A bilobed nucleus interpreted as the result of partial fusion of two daughter nuclei. Four-lobed nuclei are sometimes seen.
8. The anaphase stage of a dividing liver cell. This would probably give rise to a binucleated condition.

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phenomenon is soon followed either by a type of modified mitosis (Stough, 1931), or by amitosis (Weed, 1936) which results in a multinucleate fiber. Following injury to the definitive fiber a similar and rather extensive nuclear proliferation takes place. In the light of the occurrence of nuclear endomitosis, previously discussed, it seems possible that Stough's interpretation is tenable, that nuclear proliferation in the later stages of histogenesis of skeletal muscle is of this nature, and that only a small part of the chromatin participates in the process.

Thus we have arrived at a point where the differences between this highly modified mitosis and amitosis appear negligible from the restricted standpoint of morphology. To the geneticist this may smack of heresy. But when we consider that chromosomal multiplication, and presumably genic multiplication may take place in an apparently 'resting' nucleus, it does not transcend the bounds of imagination that even in amitosis the genes may be duplicated and the homologous pairs segregated by a process which appears so simple as to lack the apparent precision ordinarily associated with the complicated phenomenon of mitosis. Moreover, in many instances, amitotic division of the nucleus is preceded by division of the nucleolus—indirect evidence of some orderly changes occurring within the nucleus.

Thus we may encounter cases in which mitosis finds only a vestigial expression confined to relatively simple morphological changes occurring within the nucleus. This is in accord with the restricted concept of cell individuality—a nucleus exerting a sphere of influence on cytoplasm which may or may not be sharply delimited from other spheres of influence. The essential process of mitosis therefore appears to be genic duplication. These units usually are found in regular patterns in morphological entities, the chromosomes. Members of homologous pairs may be separated from one another almost immediately or may be either temporarily or permanently associated with one another in the various polyploidic states.

After this long discussion which seemed necessary to define our concepts of the several processes involved we can now return to a specific consideration of histo- and cytoteleosis and the apparent restriction of mitotic capacity in tissues and cells that occurs during this process.

Clearly, in the vertebrate neurone, mitotic capacity is lost early. As for vertebrate skeletal muscle, I have already hinted that the capacity for a vestigial expression of mitosis may persist throughout a great part of the life of this multinucleated structure.

In many epithelial tissues a germinal line with mitotic capacity persists almost indefinitely while other cells proceed to their final functional specialization. In these cases when the equilibrium of the fully grown organ is disturbed and replacements are needed, the germinal line usually responds readily and the more specialized cells usually do not exhibit mitosis. In stratified epithelia the cells of the deeper layers divide freely. In simple epithelia such as in the stomach and intestine, there are localized areas which provide for necessary cell replacements. In many glands the duct epithelium is an important source of new cells as in the mammary gland and the vertebrate pancreas.

In the pancreas the situation is complicated by its division into exocrine and endocrine components. Both the acinar cells and islet cells have usually been regarded as being finally differentiated (with the exception of the clear cell of Bensley) but recent observations raise doubts regarding the validity of this conclusion. Although cells are largely recruited from the duct epithelium, acinar cells even with zymogen granules have been observed in mitosis (Pollister, 1929). More recent observations by Woerner (1938) show that in the guinea pig after continuous injection with dextrose there is an increase in islet tissue, due in part to growth of preexisting islands by mitosis in beta cells. Quoting from Woerner:

"Moreover, there is evidence that even acinar cells may directly transform into island cells. Here are found acini among whose cells a single cell occurs which shows the properties of both acinar and beta cells. That is, a cell in the position of an acinar cell, containing a small amount of chromophile material at the base and zymogen granules toward the lumen, all characteristics of a differentiated acinar cell, at the same time is adjacent to a dilated capillary and contains specifically staining beta granules in the base of the cell toward the capillary. The mitochondria, instead of being the long filamentous type characteristic of the active acinar cell are shorter, approaching the small rod-like type, characteristic of the beta cell, but not fragmented or swollen as they are in the Mankowski cell. Such unquestionable evidence of the direct transformation of acinar cells into beta cells has not been presented before." (p. 44).

Sergeyeva (1940) also concludes "Some morphological observations suggest a possible transformation of acinous tissue into endocrine tissue and vice-versa." (Abstract). Accordingly it seems doubtful if we can, without some reservation, regard either acinar cells or islet cells as finally restricted and therefore embarked upon histoteleosis.

In the case of the liver, to which allusion has already been made, it seems very clear that in the normal adult organ, while little or no physiological growth takes place (one mitosis in 10,000 to 20,000 cells) yet the capacity of these cells to multiply by mitosis persists and whenever the stimulus is present, regeneration occurs by this method. However, these cells are usually regarded as fully restricted as to their potencies, and we can only conclude that final differentiation is completely dissociated from the restriction of mitotic capacity (Pfuhl, 1938; Steffens, 1940).

In the thyroid gland again there is no apparent residue of cells that may constitute a germinal line. All cells seem to become fully and finally differentiated and to attain an equal functional capacity. Mitotic figures are common in many adult animals (Necturus, Amblystoma; Grant, 1931 and guinea pig; Chouke, Friedman and Loeb, 1935) and no simplification of structural pattern precedes mitosis. With a suitable stimulus or under a suitably changed environment the mitotic capacity of these cells finds a complete expression.

In the case of the kidney the situation is somewhat different. In young animals (28 day old rats) mitosis occurs in the cortex with great regularity (Blumenfeld, 1938). These are apparently functional cells with fully restricted potencies but whether they are partially differentiated or just young differentiated cells it is difficult to determine. However, in older animals even in compensatory hypertrophy, following unilateral nephrectomy, hyperplasia is denied.

The case of the sensory cells of the lateral-line organ of Triturus viridescens may also be cited. During the aquatic larval period, the lateral-line system is functional. In the terrestrial or red-eft stage, the system is temporarily nonfunctional and the organs are overgrown by epidermal cells, and the sensory cells undergo some regression. Before the animals return to the water, the connections with the surface of the epidermis are regained and the sensory cells increase in number by mitosis (Dawson, 1936). These cells are finally differentiated, undergo modulation during the land-phase, and are still capable of mitotic activity under the influence of the pituitary gland which seems to be the controlling factor in inducing the appearance of the adult aquatic phase. Again final differentiation and loss of mitotic activity behave as independent phenomena.

The pituitary gland presents a somewhat different problem, owing

to the presence of chromophobic as well as granulated acidophiles and basophiles. In this gland both granulated acidophiles and basophiles as well as chromophobic elements divide mitotically and in considerable numbers. In the case of the basophiles, the activity is greatly increased in the rat by castration (Pomerat, 1940). Here even the modified basophiles, 'castration' cells, are found in mitosis. This response is not limited to young animals. In the rat other stimuli result in equally striking mitotic responses by the acidophiles. Again we must conclude that the acquisition of final functional specialization has not resulted in the loss of mitotic capacity.

In the mammalian ovary the situation is still more complex. Our chief interest focuses on the history of the follicular cells especially those composing the mural granulosa. These cells are primarily associated with the accumulation of estrogen in the follicular fluid but may produce small amounts of progesterone. Later, under the influence of the pituitary, their major function is production of progesterone. The unmodified follicular cells divide mitotically and, in the early stages of luteal transformation, mitosis (at least in the cat and rat) occurs to some extent. In the fully transformed luteal cells mitosis is rare, but can be seen in colchicine-treated rats. Only the luteal cells can be regarded as finally differentiated. Young cells retain mitotic capacity. The lack of expression of this capacity in older cells may be due to aging, to lack of the requisite stimulation, or other factors limiting the growth of this gland.

The life history of the vertebrate erythrocyte presents a striking case of histoteleosis in which morphological indications of "aging" become increasingly apparent. An early and unequivocal evidence of final restriction of this cell is the attainment of a specific functional capacity—the elaboration of hemoglobin. Unlike a glandular cell, such as an exocrine cell of the pancreas, the products of cellular synthesis are not periodically discharged but are steadily accumulated within the cell. The progressive synthesis and accumulation of hemoglobin is accompanied by modifications of the cell, characterized by a reduction in the amount of cytoplasm, regression of certain cellular constituents such as mitochondria and Golgi apparatus and even the loss of the nucleus. The cells also change in volume and acquire a specific form. These changes do not occur uniformly in all cases and proceed to different final levels in the various classes of vertebrates. (Dawson, 1932). In this instance, we are not dealing with the final differentiation of the cell but are observing only evidences and results of "work" being done by an already differentiated cell. If the hemoglobin were periodically discharged and the cell pattern reconstituted each time, all confusion would be eliminated—a cell elaboratoring

hemoglobin in such a manner would be regarded as fully differentiated.

Unless we accept the capacity to elaborate hemoglobin as a criterion of differentiation of the erythrocyte, it becomes difficult to draw the line between so-called completion of differentiation and the maturing of the cell. In the terminal stages it is obvious that maturity passes gradually into senility, with the cell denuded of many of its normal constituents. In all instances the red blood corpuscle reaches its maximum physiological importance in old age. Mitosis is common in all maturing erythrocytes. In the urodeles and embryonic chick the red blood cells divide mitotically in the blood stream even after the polychromatophilic stage is passed and typical form acquired (Dawson, 1930, 1931, 1936). Such nucleated cells when fully mature or entering upon senility apparently are not capable of giving full expression to the mitotic phenomenon, but a process ordinarily designated as amitosis is frequently seen in these cells (Charipper and Dawson, 1928).

Finally I would like to consider briefly the cartilage cell. The chondrocyte is usually regarded as differentiated or finally restricted in its potentialities and the acquisition of its specific functional capacity is readily recognized by the appearance of a specific matrix about the cell. That it undergoes certain morphological changes during histoteleosis is implied by the terms embryonal, young, and old cartilage. Typical mitoses are found in many cartilages and the grouped distribution of cartilage cells within the matrix is in part a reflection of their multiplication by this method. Ordinarily, the mitotic activity gradually ceases as the factors limiting growth become operative, but in the provision for prolongation of growth in length of the long bones an exceptional condition is encountered. Here the cells of the persisting cartilage plate immediately adjoining the epiphysis, normally proliferate rapidly and the daughter cells become aligned in rows and undergo a progressive hypertrophy. At the diaphyseal side of the cartilage plate the hypertrophied cells ordinarily break down and new bone is deposited on the remaining calcified matrix.

The rate of mitotic activity in this meristematic zone may be influenced by a variety of factors. Activity is reduced with age and ordinarily the cartilage plate disappears on the attainment of full growth. In the rat (Dawson, 1925), the cartilage plate persists at one end of the long bones even in old age, but the proliferative activity of the cells lapses. However, mitotic activity may be renewed in fully grown animals under appropriate treatment with the growth hormone of the pituitary gland. Illness, inanition (Harris, 1931; Harris and Russell, 1933), deprivation of vitamin A (Wolbach, 1937), and hypophysectomy suppress cell division, but activity may be renewed on the restoration of normal conditions.

Lack of vitamin D, however, does not suppress mitosis in the proliferative zone. The degenerative processes in the cartilage rows do not occur so extensively and the cartilage plate becomes abnormally thickened (Dodds and Cameron, 1934, 1939).

Four different fates may befall the hypertrophied cartilage cells in such rachitic conditions: (1) complete and prompt destruction; (2) simple osteoid infiltration; (3) osteoid infiltration with rejuvenation; and (4) simple cartilage rejuvenation (Dodds and Cameron, 1939). In osteoid rejuvenation, the cells near the shaft remain enclosed in their lacunae which become infiltrated with osteoid material, but preceding the infiltration the cartilage cells frequently undergo a first and sometimes a second mitosis. In cartilage rejuvenation, hypertrophied cells lying deeper within the plate regain a juvenile appearance and divide to produce at least four cells each.

Thus in cartilage cells we have numerous examples of the persistence of mitotic capacity after final differentiation and even late in histoteleosis.

In this discussion I have tried, perhaps not always consistently, to restrict the concept of cellular differentiation to the condition in which a cell is finally restricted in potency and characterized by the attainment of a specific functional capacity with the attendant morphological expression of this capacity. The later changes which follow in the life history of the cell, often involving considerable morphological modification, have been thought of as due to the process of histo- or cytoteleosis which may end only with the death of the cell. Since these changes are frequently progressive and irreversible, they cannot always be considered as modulations. By definition, modulations are reversible.

This restriction of the concept of cellular differentiation further avoids the implied necessity of using the term dedifferentiation when, under a changed environment, the cell undergoes a simplification of structural pattern without reacquiring potencies previously lost although its specific functional capacity may be suppressed or held in abeyance.

However, the formulation of definitions does not remove a practical difficulty encountered by the working embryologist or histologist. It is not always possible to determine without elaborate experimental tests when potencies are finally restricted, or when cell differentiation is completed and the process of histoteleosis is begun.

I have tried to present a broad view of cell division, especially the mitotic method, and have cited instances where mitotic capacity, sometimes greatly modified, is retained relatively late in histoteleosis. My main contention has been that the capacity for cell division is not limited by cellular differentiation per se but that other factors, some en-

vironmental, others inherent in the histoteleotic process, later limit the expression of mitotic capacity.

The greatest implication is in the situation where mitotic capacity persists apparently indefinitely, but is suppressed by environmental conditions, and finds expression only when factors favorable for mitotic division are again present. In an earlier paper (Dawson, 1937), I emphasized the fact that, in many cells, morphological simplification does not necessarily precede mitotic division. These were examples of single mitoses and it is difficult to forecast what would happen to the morphological pattern if the mitotic activity were repeated indefinitely as it is under certain conditions of tissue culture. It may be that pathologists, in some instances at least, do not need to invoke such a theory as that of "embryonic rests" to explain the morphological picture seen in neoplastic proliferation.

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DISCUSSION REMARKS

To A. B. Dawson's Paper On "Cell Division In Relation To Differentiation"

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Broadly conceived by the embryologist, the paper "Cell Division and Development" by A. B. Dawson would include such outstanding topics as (1) relationship between cell division (including cleavage) and embryonic differentiation (cleavage patterns, gastrulation, folding of germ layers, etc.), (2) cell division and morphogenesis and (3) induced effects on cell proliferation, i. e., endocrine control of growth and cell division, effects of peripheral organ-forming systems on the central nervous system, etc. Dr. Dawson considered only the first of these, to wit,—the relation of cell division to differentiation laying special emphasis upon cellular differentiation, histogenesis or cytogenesis. The main thesis of his paper was that the capacity for cell division is not limited by cellular differentiation per se but by environmental factors as well as factors intrinsic in the "histoteleotic" process. Somewhat late in the differentiation process these limit the expression of mitotic capacity. Cell division occurs in highly differentiated and functional cells including the highly specialized protozoa. Structural simplification is not essential for its occurrence.

In the discussion interest centered first on the nature of the factors which limit cell division at the terminal stage of differentiation. Both physiological age as well as genic composition appear to be important factors. In the case of genic control, size inheritance of the organism and its organs are limiting factors.

The discussion next turned to an analysis of the question whether cells during the process of regeneration or when grown *in vitro* return to the embryonic or totipotent state or may change their potency. It was generally agreed that once a cell had attained a definitive state of differentiation it may undergo a change in structural form but not a change in potency. Such a change was regarded as theoretically possible under suitable environmental conditions.

SIZE-CONTROLLING FACTORS

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The evidence concerning size-controlling factors which I shall review will be limited largely to that derived from experiments with amphibians, primarily by the method of transplantation. This limitation is partly, but not entirely arbitrary, since both the material and the method lend themselves almost uniquely to the initial study of certain phases of the question. One of the principal problems of growth is the distribution of size between the component parts of an organism. Growth acquires most of its immediate interest, after all, from the patterns or shapes it produces, more than from mere increase in mass as such. Presumably these configurations can be achieved and maintained only through the action of coordinating mechanisms which integrate the growth of the parts relative to one another. The actual operation of such devices can be readily demonstrated, however, only by disturbing the relationships which prevail in normal development. For this purpose the method of transplantation is particularly well adapted, since it is by definition a means of shifting or substituting parts. In the experiments to be reviewed here it is used, in effect, to create a potential or actual disharmony in the relative size of parts, and thereby to test for the action of factors which regulate proportionate growth. Various differentials may be invoked, such as position, when an organ or its rudiment is shifted to an unaccustomed region in the organism; growth intensity of the environment, as in heteroplastic transplantation between species of different growth rate; or of age, as in transplantation between individuals of different stages of growth.

Studies of this nature received their principal impetus from the original work of Harrison (1924), when he exchanged eye and limb rudiments between embryos of Amblystoma punctatum and A. tigrinum, and we may begin by reviewing these and subsequent experiments involving heteroplastic transplantation. Of the two species employed by Harrison and many of his followers, A. tigrinum has much the higher rate of growth. Hence an organ rudiment grafted from tigrinum to punctatum undergoes its subsequent development in an environment of lower growth intensity than that to which it is accustomed. This did not prove to be a limiting factor, however; in fact the grafted eyes and limbs not only maintained their hereditary growth rate on the slower growing hosts, but actually appeared to exceed it. Later, however, when the grafts were compared in size with control organs on better fed larvae of the donor species, a much closer correspondence was found to exist (Twitty and Schwind, 1931). Neverthe-

less, Harrison's observations threw into relief the two basic components which must inevitably be involved in the control of organ growth: first, the inherent growth potential of the organ itself, and second, the factors in the organic environment on which the expression of this potential depends. One would not expect a given organ to find precisely the same conditions for growth on all species to which it might be grafted, nor that the envionmental conditions involved are necessarily the same for different organs. It is true that eyes and limbs grafted between A. punctatum and A. tigrinum reduplicate essentially their normal growth rate and we might conclude that insofar as these organs are concerned the environmental conditions in the two species of Amblystoma are virtually identical. But, if anything, this situation is exceptional. Certain other organs grafted between these two species do not follow the same rules, nor are the results necessarily the same when the eyes and limbs themselves are exchanged in other species combinations (Twitty and Elliott, 1934). Even when a slightly different race or strain of tigrinum is substituted, the results of limb transplantation between it and punctatum seem to differ from that described above (Detwiler, 1930). In many cases, however, these differences in results are more illuminating than disconcerting. Insofar as they do not defy interpretation completely, they serve to extend, rather than to obscure, our knowledge of the factors which influence organ size. Since most of the literature in this field has been cited elsewhere (Harrison, 1933; Twitty, 1934), I shall confine myself principally to selected examples illustrative of the types of situations encountered.

Two of the most striking instances of size-regulation are afforded by the results of exchanging heart rudiments and portions of the central nervous system between the two species of Amblystoma. For a brief period during earlier larval growth the grafted hearts (Copenhaver, 1930, 1939) follow their genetic trend and hence become either "too large" or "too small" for their respective hosts. Instead of continuing in this manner, however, as would the eyes and limbs, they soon begin to adjust their size to that of their hosts, and eventually assume proportions entirely appropriate to the animals on which they are growing. This may be interpreted as a response to the functional demands imposed upon them by their hosts, although of course the mechanism for such adjustment is not clearly understood. In any case, it is not surprising that they do not follow the same rules as the eyes and limbs. The latter play no crucial role in the essential machinery of the organism, and are freer to express their growth capacities independently of the scale or requirements of the rest of the animal. The results with the spinal cord (Detwiler, 1932) are of similar nature, except that here the tigrinum graft on punctatum, for example, even exceeds its normal growth rate for a time. The striking disproportion which thus develops

is only temporary, however, and as with the heart, subsequent retardation of growth eventually results in complete size adjustment. Regulation in this case is possibly attributable to the factors known from other experiments to influence cell number in the spinal cord, including the number of axones reaching the graft from other levels of the cord. Incidentally, the study of size-control in the nervous system is an entire chapter in itself, which I shall make no attempt to cover in this review (see Detwiler, 1936; Harrison, 1935).

The ear is another organ offering an example of size regulation, although the adjustment is not as great as in the cases just cited. The inner ear of punctatum developing upon the larger tigrinum expands beyond its normal size, but not to the dimensions appropriate for the host, and the reciprocal experiment gives a corresponding result (Richardson, 1932). The ear is a delicate, thin-walled structure, and it was suggested that mechanical limitations of space imposed by the surrounding muscles and skeleton may affect its size.

I shall interpose at this point reference to certain experiments which might perhaps have been used as a starting point for the survey, since they deal with transplantations in even earlier stages of development than those I have just mentioned. They deal not so much with subsequent growth rate as with the factors which determine the initial size of organ anlagen, at the time of their first delineation in the embryo. An example is afforded by Rotmann's (1939) experiments with the eye vesicle, using two species of European Triton, T. taeniatus and T. cristatus. Of the two embryos, cristatus is the larger, and its eye vesicle is correspondingly larger when it first forms. We know that it is first determined as retinal material while it is still a portion of the open medullary plate, and hence before it actually evaginates to form a vesicle. Thus if a piece of ectoderm from some other region, say ectoderm ordinarily destined to form flank skin, is grafted into this region at a sufficiently early stage it will subsequently form the eye vesicle. One of the possibilities considered by Rotmann is that the area of retinal material thus determined, and hence the initial size of the eye vesicle, is arbitrarily proscribed by the inductor itself. An alternative is that the inductor merely releases retinal-forming potentialities, and that the actual scale of the rudiment is an independent expression of genetic qualities in the reacting tissue. This was tested by grafting gastrula ectoderm of cristatus in place of the future eye region of the smaller taeniatus embryo. The eye vesicles later forming from the grafts tended to conform in size to that of the host, suggesting that the inductor not only "determines" the retinal material as such, but also specifies its scale. In a few cases, however, the vesicle was larger than that of its host; and in the reciprocal experiment taeniatus ectoderm on

the larger cristatus formed a vesicle appropriate in size for the donor species, that is, much smaller than the normal host vesicle.

Incidentally, initial conformity of the optic vesicle to the size prescribed by the host, as in the first combination, does not mean that its subsequent growth rate and eventual size is similarily altered. Rotmann's experiment was performed independently by the writer several years ago (unpublished), and it was found that such vesicles, although starting at the same size, later grew into eyes considerably larger than the normal organs of the host. This is in accordance with the more rapid larval growth rate of the donor species.

Rotmann (1939) has obtained a more positive answer to the question of initial size in similar experiments with the lens. Here the scale of the rudiment is apparently an expression of genetic qualities in the lens forming tissue itself. Thus cristatus ectoderm grafted over an optic vesicle of taeniatus forms a lens which already adheres to the cristatus scale at its earliest recognizable stage of development. This lens was formed at the behest of the small taeniatus eye vesicle, but apparently the "command" did not include specifications concerning the size of the structure elicited. Corresponding results were obtained in experiments with the "balancer" (Rotmann, 1935a) and the epidermal covering of the external gills (Rotmann, 1935b).

The results just described for the lens raises a further question which is of even greater interest for our present discussion. A chimaeric eye is created in which the lens and optic cup are of disharmonious proportions, and we may inquire what the consequences will be during subsequent growth of such an organ. This question has also been studied by Rotmann, but it was first tested by Harrison (1929) in experiments with the two species of Amblystoma. Optic cup and lens are much larger in tigrinum than in punctatum, so that when the optic vesicle of one and the lens epidermis of the other are combined into a single eye a potential maladjustment of considerable magnitude is created. This does not materialize, however. Optic cup and lens exert a mutual influence on the growth of one another, with the result that the chimaeric eye eventually displays harmonious proportions. For example, a tigrinum cup, when combined with a punctatum lens, does not grow as large as it normally would, while in turn the lens exceeds its usual dimensions. These results have been confirmed and extended by Ballard (1939).

We have here an example wherein the growth intensity of one structure markedly affects that of another. One may inquire why this is not the case when the eye is grafted intact from one species to the other. A tigrinum eye transplanted to punctatum is placed in an environment of much lower growth intensity than that to which it is accustomed, and

yet its size is not significantly affected. The explanation probably lies in the fact that the eye as a whole develops as a relatively self-contained and independent entity. This is not true, however, of the relationship between its component parts. The retina and the lens are intimately engaged throughout their development. Numerous examples of local size adjustments between parts intricately related during their development could be offered, including further instances drawn from the eye itself. Both the cartilaginous capsule investing the eye ball and the extrinsic ocular muscles partly adjust their size to that of the bulb (Twitty, 1932). The eye lids, which appear first at metamorphosis in Amphibia, are apparently independent of such influences. Thus tigrinum cornea grafted over a punctatum eye ball gives rise to lids much too large for the bulb itself (Ballard, 1939). An interesting example within this category of local effects is provided by Rotmann's (1935b) experiments with the external gills. When the gill epidermis is replaced by that from a more rapidly growing species, the graft causes the underlying branchial vessels to increase their diameter even before blood flow is established.

The mechanism underlying these local size adjustments is not understood, and there is no basis for assuming it to be the same in all instances cited. Huxley (1932) has developed the concept of "growth gradients" to account for essentially similar growth relationships deducible from measurements of crustacean appendages, etc. If we are to apply this designation, one of the most striking instances which could be offered is provided by the results of Stone (1934). Using embryos of punctatum and tigrinum, he joined the anterior half of one species with the posterior half of the other. In the subsequent growth of these chimaeras the pace was apparently set by the anterior half, with the posterior portion adjusting its growth to form a larval of harmonious proportions. Further experimentation will be required before one can attempt to interpret or classify this important result. Each half of the chimaera is in itself very complex, involving many organ systems, and the total effect may represent the summation of several contributing influences comparable to those encountered in experiments with single organs.

How then can we summarize up to this point the factors which regulate proportionate organ size? In extreme cases, such as the heart, a single factor, namely function, might seem to provide the only mechanism needed to insure ultimate size-adjustment. In others, however, no such simple situation exists, and we may turn again to the eye for illustration. Ignoring the factors involved in determining initial size of its rudiment, the eye, once formed, is an organ with a strong capacity to express its genetic growth rate independently of the growth intensity of the rest of the animal. Thus in heteroplastic transplantation it may be-

come markedly disproportionate in size. It is true that its total size is partly a product of mutual growth influences between its parts but this is an adjustment within itself, not with reference to the rest of the organism.

Now until this point we have ignored one differential of first importance, namely that of age, or stage of growth. We have said that a tigrinum eye grafted to punctatum grows to a size much "too large" for its host. It must be kept in mind, however, that although this is true of its absolute size, it is not true of its stage of growth. The latter is in fact appropriate for the host, since at comparable ages a tigrinum larva, including its eye, is much larger than that of punctatum. So that when we say a tigrinum graft is "too large" for its host, we are likely to obscure the fact that so far as growth stage is concerned there may actually be a nice adjustment between graft and host.

As before, the regulation of harmonious growth stage can be studied only by creating an initial discrepancy in this respect between the part and the whole. Embryonic transplantation is not entirely suitable for this purpose, since it it difficult to secure a sufficient age differential between donor and host. Larval stages were accordingly used instead, and eyes were grafted between younger and older individuals of the same or different species (Twitty, 1930; Twitty and Elliott, 1934). When both donor and host were of the same species, they were of course of different size. When of different species, for example A. punctatum and A. tigrinum, donors and hosts of the same size could be employed, since it follows from the difference in growth rate between the two species that a tigrinum larva of a given length is considerably younger, that is, less advanced in growth, than a punctatum of identical length. In either event, transplantation resulted in eyes which were either "too small" or "too large" relative to the growth stage of the host. This initial disproportion did not persist. It was regulated during subsequent growth of the hosts by accelerated or decelerated growth of the transplants.[1]

In an effort to interpret these results, we may inspect a given experiment, in which the eye is grafted from a smaller to a larger larva of the same species. The graft starts with an initial handicap, but it subsequently "catches up" with the normal eye of the host. It was at first believed that this regulative growth might be interpreted by (1) postulating a difference in the assimilative capacities of the graft and host, and by (2) invoking the principle of competition.

That the transplants have a higher assimilative capacity than their

[1]Similar results have been obtained recently by Bodenstein (1940) following transplantation of eye and limb discs between younger and older larvae of Drosophila.

older hosts seems particularly clear from the following experiment. The transplantation was identical to the one just mentioned, but instead of feeding the host as before, it was starved from the time of transplantation or even earlier (Twitty and DeLanney, 1939). As a consequence of the starvation, neither the host nor its normal eye increased in size. In fact they tended to diminish slightly. In contrast to this, the younger, i. e., the grafted eye, was able to accomplish a very significant increase in size. Both eyes are nourished from a common blood stream, so that both presumably have equal access to the food continuing in circulation under imposed starvation. Yet it is only the younger organ which is capable of obtaining materials for growth. There thus seems to be a decrease in the assimilative ability of the eye, and probably other organs as well, as the animal increases in age and size. This assumption is not unlikely, and is possibly supported by the accepted fact that growth activity, as measured by percentage increase, tends to diminish as growth proceeds.

If we accept this evidence one could reason as follows. When an organ is grafted to an older animal, it is placed in competition with tissues characterized by a lower ability to obtain food from a common circulation than those of the animal from which it was taken. Given this advantage, which it formerly lacked, it could secure a larger share of food than before and thereby exceed its original growth. In order to establish this view, however, it is necessary to demonstrate that food is actually partitioned in the growing animal on a competitive basis. And the only evidence available does not seem to support such an idea.

The experiments bearing on this question were designed to test the possibility that when two young organs are undergoing size regulation simultaneously on the same animal, each might limit the degree of regulation achieved by the other. First, however, it is necessary to explain that regenerating organs seem to follow the same rules as transplants undergoing size-regulation on an older host. When the tail, for example, is amputated, a bud or blastema is formed which grows rapidly to approximately normal proportions. Such regeneration buds have been shown to have certain properties of embryonic organs (Schotte, 1939), and it is entirely possible that their greater physiological youth, as compared with that of the rest of the animal, is a factor in their regenerative growth. In any event, the tail blastema, like younger transplants, can undergo regulation into a complete tail even under complete starvation, and do so repeatedly after successive amputation. This fact made it possible to construct the following experiment (Twitty and DeLanney, 1939).

The eye of a salamander larva was replaced by one from a younger animal, followed in turn by amputation of the tail of the host. The

larva was completely starved during the course of the experiment. Under this adverse regime one would expect competition for available food to be as keen as under any which is likely to occur, and it was conceived that the regeneration of the tail might create a scarcity of available food which would limit the size regulation of the grafted eye. This proved not to be the case however, So far as could be ascertained the graft accomplished the same amount of growth as in control series where the tail was left intact.

This is perhaps not conclusive evidence that food is partitioned on a non-competitive basis, but it argues strongly against competition being a potent factor, especially under optimal conditions of growth when food is not limited.

If we eliminate this concept, another approach must be made to the interpretation of size-regulation in organs grafted between younger and older animals. The greater assimilative capacity of young transplants will explain why they grow more rapidly than the corresponding organs of the host. It will not in itself explain, however, why they grow more rapidly than they would have in their original environment, which is essential if they are actually to overtake the host organs while the latter are still growing.

In meeting this difficulty we may possibly obtain a clue from one of the experiments already described, namely the transplantation of an eye to an older, starved host. The graft grows while the size of the host and its normal eye remains constant, and we have interpreted this as evidence that the graft has a superior ability to obtain food from the common blood stream. If it is not expecting too much of a single experiment, however, it may prove or strongly suggest another fact as well. This is seen when we compare the behavior of the graft, not with the normal eye of the host as before, but with what its own behavior would have been if left on the donor animal. Assuming that this donor had been starved, like the host in the experiment, the eye in question would have of course remained static in size or have even become smaller. We see thus that in grafting it from a younger to an older animal we have improved its opportunities for growth. If this advantage cannot be ascribed to competitive interplay, the simplest alternative which remains is that the basic level of nutrients is higher in the blood of the older larva than in that of the younger.

As we shall see later, such an increase in nutrient level with age, together with an accompanying decrease in assimilative capacity of the tissues themselves, would afford a satisfactory means of interpreting the results observed. In fact, some such reciprocal set of changes in the cells on the one hand and in their environment on the other would seem to afford almost the only suitable way of accounting for the facts.

This conception was first proposed entirely by inference from the experiments already described. And in fact no proof is as yet available. Recently, however, certain additional evidence has been obtained which is at least in agreement with such an idea.

This consists in a series of chemical determinations on blood from A. tigrinum larvae of different ages, made by Dr. W. J. van Wagtendonk of Stanford University in collaboration with the writer. These results

TABLE I

BLOOD ANALYSIS OF AMBLYSTOMA LARVAE

(ALL DATA IN MG. PER CC. OF BLOOD)

Avg. Length of Larva in mm.	Total N		Non Protein N		Amino acid N		Sugar	
		Average		Average		Average		Average
44	17.20	17.00	3.00	2.90			1.72	1.78
	16.80		2.80				1.84	
45	18.40	18.40	2.88	2.88			1.76	1.76
	——		——				1.76	
47	16.40	16.63	2.74	2.85			1.73	1.73
	16.85		2.96				1.73	
50	17.85	17.72	3.15	3.20			1.80	1.75
	17.40		3.26				1.70	
55	17.10	17.03	3.33	3.29	1.84	1.88		
	16.95		3.24		1.91			
56	16.80	16.97	4.41	4.62			1.50	1.56
	17.13		4.83				1.62	
70	16.40	16.40	6.76	6.76			1.40	1.40
	——		6.76				1.40	
72	16.35	16.35	11.76	11.45			1.73	1.73
	16.35		11.13				1.73	
85	16.90	16.80	11.20	11.30	9.54	9.34	1.76	1.76
	16.70		11.40		9.14		1.76	

Milligrams per cubic centimeter of total nitrogen, non-protein nitrogen, amino acid nitrogen, and sugar in blood drawn from larvae of Amblystoma tigrinum of various sizes. The blood was taken by micropipette directly from the heart, several larvae usually being required to supply a volume adequate for analysis. Each sample was divided into a number of portions sufficient to permit, in most cases, two independent determinations of each constituent. The larvae were reared in the laboratory from eggs collected near Nashville, Tenn., by Dr. Karl Mason. Feeding was carefully controlled to avoid any complicating effects of type or quantity of food on the results. Detailed discussion of the data and description of chemical methods employed will be given in a separate publication.

are still incomplete and are based principally on measurements of only three constituents, namely, total nitrogen, non-protein nitrogen, and sugar; amino acid nitrogen was determined directly in only two blood samples. Blood from several different size groups, ranging from 44 mm. to 85 mm. in body length, was used for the determinations. Attempts to obtain adequate volumes of blood from smaller larvae have so far been unsuccessful. A summary of the data is given in the accompanying table.

The essence of the results is as follows: (1) The concentration of total blood nitrogen remains essentially constant. (2) The non-protein nitrogen shows a marked rise. Although this fraction includes other sources of nitrogen, such as ammonium compounds and urea, the principal increment with age appears to be in amino acid nitrogen, judging from the two tests where the latter was measured directly. (3) Since the values for total nitrogen comprise both protein and non-protein nitrogen, it follows that the protein nitrogen decreases in concentration as the non-protein nitrogen rises. (4) The concentration of blood sugar shows little change.

The significance of these data for our present purposes depends upon which of the various blood components is most crucial for the synthetic processes of growth. If proteins as such were the principal building material utilized, the evidence obtained would suggest a decline with age, instead of postulated improvement, in the nutritive opportunities afforded by the blood stream. If on the other hand, as would seem more probable, amino acids are the principal requisite, especially during larval growth when synthesis of new protoplasm is proceeding at such an active pace, the evidence tends to offer strong substantiation for our hypothesis.

We may thus present as briefly as possible the following conception of the manner in which the growth of an organ is geared with that of the rest of the animal. The eye will be used as an example, although the same rules probably hold for other organs and tissues except as they may be influenced by superimposed factors of function, mechanical limitations of space, etc. As the eye increases in size and physiological age its cells suffer a gradual loss in assimilative capacity. This is accompanied by a gradual improvement in the nutritive opportunities afforded them by the blood stream. These two sets of changes together provide an effective means of insuring constant size-adjustment. The size of the eye at any given moment is a function of its own assimilative capacity and the nutritive opportunities afforded by its environment at that particular stage of growth. Thus in the course of normal development a state of equilibrium is always in effect. If now the eye is placed on an older larva, this equilibrium is disturbed, since the graft is ex-

posed to a richer medium for growth than that to which it was previously adjusted. Growth acceleration ensues, and appropriate size relationships are restored. It is important to bear in mind, however, that a nutritive differential alone would not account for this result. The richer environment enables the graft to exceed its normal growth rate, which is of course an essential feature of the picture. But it is equally important, once the size handicap has been thereby overcome, that this acceleration should be halted. Otherwise the graft would not only overtake the normal organ of the host, but actually surpass it in size. In other words, size-regulation is achieved by a temporary advantage of the transplant, but would be defeated if this advantage were permanent.

This brings out clearly the indispensable role of the other component involved in growth regulation, namely the decreasing assimilative capacity of the cells themselves. Growth acceleration enables the transplant to overtake the host, but this very acceleration likewise speeds the decrease in its assimilative capacity to the point where its growth advantage disappears, that is, to the same level as the rest of the animal. When this condition is reached, equilibrium is restored. The same mechanisms continue to operate, of course, and it is to them that the size relationships which we observe in normal development are probably to be ascribed.

As stated above, I believe it will be difficult to offer an interpretation of size-regulation without postulating some such dual mechanism as that proposed here, however the changes in the cells and those in their organic environment may be visualized or identified. The problem is obviously one which calls for further chemical and physiological studies.

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THE PATHOLOGY OF DEVELOPMENT

(Abstract)

By

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One phase of an extensive constitutional study under progress in this laboratory has been an investigation of spontaneous neoplasia in the rabbit and, as a result of routine clinical and pathological examinations carried on over a period of years, a considerable series of tumors has been found. Cancers of the breast and of the uterus have occurred with the greatest frequency and have been subjected to more thorough study than the less common tumors of other organs. It is encouraging that in morphology, mode of development and biological characteristics, these growths bear a much closer resemblance to human cancers than do the corresponding tumors under study in other animal species.

Cancers of the uterus occur with the highest incidence in families of rabbits known to transmit hereditary abnormalities of the endocrine system. They are also found scattered throughout the general population, but in both situations their occurrence is limited to animals that have recovered from an attack of toxemia of pregnancy (1). An investigation of this condition suggested that it arose from a disorder of internal secretion and experimental studies in which the disease was reproduced by the administration of pituitary extracts furnished confirmatory evidence (2, 3, 4). Such observations indicated that the origin of the uterine tumors might also be associated with disturbances of internal secretion. Examination of the endocrine organs of effected animals showed the constant presence of lesions which were identical with those produced in mice by the administration of estrone and it was therefore assumed that the spontaneous tumors represented a natural analogue to the experimental induction of neoplasia with such substances. The occurrence of early neoplastic changes in animals that have recovered from experimentally induced toxemia of pregnancy confirms the existence of the relationship observed clinically and further investigations are in progress.

The incidence of breast cancer is limited to two family lines of rabbits and the characteristic growths differ in mode of development and in microscopic appearance (5, 6 and 7). In one family, the tumors arise in normal breast tissue and are characterized by an acinar-like structure. In the second family, tumor formation is preceded by a disorder of the breast which is clinically and pathologically identical with chronic cystic mastitis in women and the growths are characterized by a papillary type structure. The early stages of tumor development

in both families are preceded by reproductive disturbances and, at autopsy, changes are found in the endocrine system which are identical in nature with those observed in animals bearing uterine tumors. It seems probable, therefore, that a common aetiological factor is concerned in both uterine and breast cancers and that the preponderance of breast growths in certain genetic lines and of uterine growths in others is due to the operation of hereditary location factors.

A characteristic feature of the breast and uterine cancer is the occurrence of a well defined developmental history, They do not arise as a sudden transition of normal cells but, on the other hand, represent the final step in a progressive developmental process during the course of which the primary neoplastic focus passes through successive stages of anaplastic cellular change, local tissue invasion, foreign tissue invasion and eventually metastasis. Transplantation experiments utilizing the anterior chamber of the eye as an inoculation site showed that the tumors could not be transferred to normal animals during stages of anaplastic cellular change or of local tissue invasion but could be transferred to normal animals during the stage of foreign tissue invasion (8). It was, therefore, concluded that anaplastic cellular change or local tissue invasion in the primary growths of these tumors did not constitute evidence of autonomy but rather stages in the development of autonomy, the final attainment of which was only evidenced by foreign tissue invasion.

In contrast to the failure of transfer to normal animals during stages prior to foreign tissue invasion, it was found that at such stages the developing tumors could be successfully transplanted to animals bearing spontaneous growths. This finding suggested that special factors were present in the spontaneous hosts but absent in normal animals and the nature of the endocrine changes constantly found in tumor bearing animals indicated that one of these factors might be an abnormal secretion of estrone. It was subsequently found that dependent tumors survived and grew in estrinized animals, whereas early death of the transplants occurred in normal animals used as controls and it was concluded that the constitutional status incident to an abnormal secretion of estrone was one of the factors essential to the continued growth and development of the tumors (9).

Cancers of the breast and uterus have been carried for many serial generations in normal rabbits and experimental investigations have been concerned with attempts to alter malignancy and to determine the immunological status of animals during different stages of tumor growth (8, 10). The tumors have also been successfully transplanted to foreign species, including guinea pigs, hens, goats, sheep and hogs, by means of the anterior chamber technique (11). The tumors receive

a blood supply from the alien host and grow progressively. The cells of the transplant, however, are descendants of the original tumor and not derivatives of the foreign species.

It has also been possible to transplant human tumors to lower species using this route of inoculation (11). A considerable series of human tumors have been tested and it has been found that successful transplantation to normal animals could only be performed after the occurrence of foreign tissue invasion and it was concluded that, in human as in rabbit tumors, this stage marks the attainment of autonomy.

The primary conclusions indicated by the findings described in this abstract are first, that the rabbit cancers are not simply local tissue diseases but, on the other hand, represent local manifestations of a generalized constitutional disorder and, second, that in the rabbit the primary neoplastic focus is not a cancer and that before becoming a cancer it must undergo a process of progressive evolutionary development. Development to cancer will not take place in normal animals but is dependent on a special constitutional status which may be evoked experimentally by the administration of estrogenic substances. There are indications that human cancers undergo a similar developmental course in their advance to cancer and further studies are in progress.

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DISCUSSION REMARKS

To H. S. N. Greene's Paper On "The Pathology of Development"

By

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Pathology, being an applied subject, is an art much more than it is a science. Therefore, it has in it many qualitative terms, many rule of thumb procedures, much of it is done on the basis of experience and impressions. Its science is in biology and therefore pathologists are constantly on the look-out for new biological principles and facts which they may seize and apply to their problems.

It must be remembered too that most pathologists deal with human disease and the human element entering into the picture gives to the subject a quality of urge and necessity which often drives pathologists to what seem like extremes. Sometimes they rush in where biologists fear to tread. Most of us would like to pass on just a bit of this pressure to biologists so that they may become more aware of our problems and many times, more than they do, come to our rescue.

Pathology deals in general with three major subdivisions (1) degenerations, infiltrations and a host of similar lesions; (2) all the different kinds of inflammations with their specific and non-specific manifestations, and (3) the big subject of tumor formation.

Most pathologists recognize that certainly the last two of these subdivisions, and sometimes some of the first, belong to the growth problem. It will not be possible to discuss or even mention all the phases of the pathologist's every-day work in which growth problems appear so just a few will be told.

Many times pathologists are called upon to predict. This requires a knowledge of statistical approximations in their subject but it also involves, since they deal with individuals, a peculiar knowledge of cell behavior on which to base their predictions. An ever-present question is this—asked by clinicians, patients and relatives alike—"shall I have a certain growth removed because it will turn into cancer"? This "precancer" diagnosis is a prediction. I leave to the biologist the implications in an answer to such questions.

As Dr. Greene has so admirably brought out, the pathologist is interested in "constitutional differences". He wonders why some patients develop keloids and excessive adhesions; he agrees with Virchow that some people have a "connective tissue diathesis". Practically, he does

not care if the scar from an appendectomy results in a keloid nor does the patient, unless it is a girl who happens to be in the chorus, but it does matter in injuries of the face so frequent in automobile accidents, in the scars of thyroidectomies and similar affairs. Do these ever turn into malignancies is a most important question confronting him daily.

The pathologist is also interested in the differentiation of cells for many reasons. To give one—when the cells of an embryonal adenocarcinoma of the testis reach a stage of differentiation wherein they produce hormones, he not only can diagnose the tumor of the testis by finding the hormones in the patient's urine but he can guide the amount of after-treatment by the very same procedures.

We pathologists have been twitted many times about nomenclature, especially that of tumors. We realize that many of the older names can and should be tossed over-board and we are anxious to do it, but we will do it only as biologists give us good sound reasons for changing names which have become familiar to us. Will they settle for us the important question of whether individual cells, once they have differentiated to certain degrees, can de-differentiate? Will they settle for us the question of how far can a cell differentiate until it can no longer divide? Answers to problems like these will give us opportunity for a house-cleaning in more ways than one.

So I could go on with problem after problem but I will close by repeating a thought expressed above—please, biologists of all kinds, enter with us into our problems, help us to carry the load of application to hundreds of thousands of tumor cases yearly, to say nothing of the thousands of other patients. In return we hope we will continue as we have at times in the past, giving information as to normal processes from studies of the abnormal.

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THE METHOD AND THEORIES OF PHYSICAL SCIENCE IN THEIR BEARING UPON BIOLOGICAL ORGANIZATION

By

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I
INTRODUCTION

By the organization of a living creature we mean its relatedness both morphological and physiological. Not merely the distribution of material at any instant of growth, but the entire process of growth itself, together with the subsequent maturity and senility, is organized. The problem of growth may be regarded therefore as a special case of the more general and fundamental problem of organization.

This approach has certain advantages. It permits us to bring to bear upon our understanding of the particular phenomenon of growth, all that we have learned from many sources concerning the factors and principles involved in the underlying problem of organization. Also, it prevents us, in a way that we shall see to be important, from attributing to factors unearthed by a direct attack on the problem of growth, a greater significance than they deserve, due to the neglect of other equally important factors in organization, solely because the latter do not come within the purview of the experimental investigation under consideration. In addition, it enables us to discover the insufficiency of certain concepts and theories we are using, even when these conceptions have experimental evidence in their support, by enabling us to deduce from them consequences which reveal their empirical shortcomings as a complete solution of the problem. An example will appear in the sequel in our consideration of the thermo-dynamical theory of organization. By establishing the insufficiency of traditional theories, the theoretical approach makes us aware of the need for a new hypothesis and often indicates the character which it must possess, thereby guiding one to new modes of experimental investigation and to the actual empirical discovery of new evidence. This also will be exemplified at the end of this paper.

These advantages are possible, however, only if we appreciate the rôle of theory, concepts, and deductive logic, as well as of inductive observation and experimentation in natural science, and master the scientific method for relating deductively-formulated theory to inductively- and experimentally-controlled data in a trustworthy manner. This method has been developed over the centuries by the physical and mathematical sciences. It is much more complicated than has usually been supposed.

II

THE METHOD OF THE MATHEMATICAL AND PHYSICAL SCIENCES

A. *Two Stages in the Development of an Empirical Science.*

The history of science shows that any empirical science in its normal healthy development begins with a more purely inductive emphasis, in which the empirical data of its subject matter are systematically gathered, and then comes to maturity with deductively-formulated theory in which formal logic and mathematics play a most significant part. Geometry, for instance, began with the earth measurements of the ancient Egyptians and early Greeks and came to maturity in the deductively-formulated *Elements* of Euclid. Physics took the whole of the Greek period and the entire Middle Ages to develop its early natural history phase, and passed to deductively-formulated theory when Galileo discovered its new key primitive concepts and Newton, taking Euclid's geometry as his model, generalized Galileo's concepts and developed them systematically in the deductive theory for mechanics which constitutes his famous *Principia*. These two stages in the normal healthy development of an empirical science we shall term (a) the natural history stage and (b) the stage of postulationally prescribed theory.

It appears that biology is at present struggling with the transition from the first of these stages to the second. It is nearing the completion of the systematic observation, description, and classification of living creatures, both in their naturally and experimentally observed manifestations, which Aristotle inaugurated in his founding of the science. Many signs are present that it is now passing to the stage of deductively-formulated theory. Mendel's and especially Morgan's work on heredity forms such a theory for a special branch of the science. The formal, deductive character of Woodger's work (1937) is self-evident. Rashevsky's application (1938) of mathematics to specific physiological processes is a third striking example.

There are, however, other evidences prevalent in the work of practically every biological scientist. They have exhibited themselves again and again throughout the papers of this symposium. I refer to the use of the entities, concepts, and principles of chemistry and physics in the elucidation and understanding of biological processes. More and more biologists are leaving purely natural history biological concepts for those of physics and chemistry in their experimental approach to and scientific analysis of living systems. This is now a commonplace. Its significance in the development of biology is not, however, so fully appreciated.

This significance arises from the fact that in passing from the purely biological concepts of the early stage of development of the science to the concepts of physics and chemistry biologists are carrying their science from the natural history stage to the stage of deductively-formulated theory.

The reason for this is that whereas the concepts of the natural history stage refer to directly-observable factors such as the shapes, colors, etc., of living creatures, the protein molecules, hydrogen ion concentrations, etc. of physics and chemistry are scientific objects or systems which are not directly apprehendable, but are, instead, unobserved, postulated entities known only by theory and consequently requiring a fundamentally different scientific method for their trustworthy treatment than the method in which biologists are trained in the natural history stage.

This point can be put more precisely by saying that in the passage of a science from the natural history stage of its development to that of deductively-formulated theory a fundamentally new type of scientific concept appears which entails, in turn, a radically different type of scientific method. This means that the kind of scientific training which is adequate for the natural history stage is necessary but quite insufficient when the science passes to deductively formulated theory. The nature of the two stages of this development will make this clear and also throw light on the difference between the two types of scientific concept for the two stages.

At the beginning of any empirical science, its obvious task is to determine the natural history materials of its subject matter. It must gather the data for which the deductively-formulated theory of the second stage is to account. Consequently, the method of the science in its natural history stage is predominately inductive. The emphasis is upon immediate apprehension and observation. The scientist's task is to meticulously observe, describe, and classify.

Because of this a certain type of concept arises in the natural history stage. We shall term it a concept by inspection. *A concept by inspection is one the complete meaning of which is given by something immediately observable.* Thus when Linnaeus described flowers in terms of the color and shape of their petals, he was formulating a biological science in the natural history stage in terms of concepts by inspection. The papers in this symposium which presented pictures of the structure of cancer or the shape of the salamander resort to the same type of concept. 'Blue' in the sense of the immediately-seen colour is another example.

To be sure, even in this natural history stage with its preponderant emphasis on inductive science, one does not have "pure fact" apart from all concepts and theory. The popular fallacy that science is concerned

only with facts and has nothing to do with concepts and theory rests upon the failure to draw a distinction between what may be termed observed fact and described fact. "Observed fact" may be defined as that which is immediately apprehended apart from all concepts and theory; "described fact" is observed fact brought under concepts and hence under theory. To have purely observed fact apart from all concepts a scientist could merely stare at his data and never report his observations in scientific meetings and in scientific journals. The moment he does the latter, he has described as well as observed, and thus introduced concepts in addition to mere fact.

Furthermore, every description throws individually-observed data into classes with respect to some abstracted character. These classes fall within each other as species within genera. Since Aristotle, it has been realized, as Professor Whitehead in our own time has repeatedly emphasized, that there are many possible modes of abstraction from the same observed data, defining many possible principles of classification. Thus it becomes inevitable, even in the most emphatically inductive natural history type of science, that inescapable elements of theory are present. Charles Darwin, whose work and theory belong exclusively to the natural history stage in the development of biology, says, "How odd it is that anyone should not see that all observations must be for or against some view, if it is to be of any service." Even natural history science, using only concepts by inspection, involves concepts and theory.

It also involves formal logic. Its method of classification, including species within genera, has implicit in it the logical relation of class inclusion. From this relation the syllogistic deductive logic of Aristotle and the logic of classes of Boole can be developed. Thus even the natural history stage of any empirical science involves theory and deductive logic as well as directly apprehended fact and induction. This is fortunate; otherwise natural history theories, such as Darwin's theory of evolution, which assert more than is immediately observable, could never be put to an empirical test by appeal to their deductive consequences.

None the less, scientific theories in the natural history stage are inductive to a degree and in a sense which is not true of theories in the second stage of development of a science. This happens because the theories of the natural history stage are developed entirely in terms of concepts by inspection. What each concept in the theory means is given by something directly apprehendable.

In the second stage of a science an entirely different type of concept is introduced. The reason for this, in physical science, has been stated clearly by Professor P. A. M. Dirac (1931), at the beginning of his

famous paper on generalized quantum mechanics. He writes, "The steady progress of physics requires for its theoretical formulation a mathematics that gets continually more advanced. This is only natural and to be expected. What, however, was not expected by the scientific workers of the last century was the particular form that the line of advancement of the mathematics would take, namely, it was expected that the mathematics would get more and more complicated, but would rest on a permanent basis of axioms and definitions, while actually the modern physical developments have required a mathematics that continually shifts its foundations and gets more abstract." In short, it is not merely by accumulating more experimental knowledge in terms of old, theoretical concepts but by changing the basic theoretical concepts in terms of which both old and new experimental knowledge is expressed and understood that physics has advanced. Professor Dirac adds, "There are at present fundamental problems in theoretical physics awaiting solution, *e. g.*, the relativistic formulation of quantum mechancis and the nature of atomic nuclei (to be followed by more difficult ones such as the problem of life), the solution of which problems will presumably require a more drastic revision of our fundamental concepts than any that have gone before. Quite likely these changes will be so great that it will be beyond the power of human intelligence to get the necessary new ideas by direct attempts to formulate the experimental data in mathematical terms."

This last sentence explains why empirical science introduces a new type of concept in passing from the natural history stage to that of deductively-formulated theory. The reason is that concepts by inspection referring to directly observable factors ("direct attempts to formulate the experimental data") turn out to be inadequate to account systematically for all the observable data. With the passage of time and the accumulation of evidence, the empirical scientist finds it impossible to account for his natural history subject-matter in terms of directly observable factors and their relations. Recourse, in the construction of adequate, empirical, scientific theory, has to be made to unobservable, postulated entities and factors rather than to directly inspectable items. Thus a fundamentally new type of scientific concept appears. We shall term it a concept by postulation. 'Electron' and 'blue' in the sense of the number for the wave-length in electro-magnetics, are examples.

Its definition is important. *A concept by postulation is one the meaning of which is proposed for it by the postulates of the deductive theory in which it occurs.* The crucial factor to note in this definition is that one does not determine the meaning of a concept by postulation by inspecting something which is directly observable; instead, one must examine the postulates of the deductive theory in which the concept

occurs. Apart from these postulates, such a concept is a meaningless mark. For example, in Newton's mechanics a scientist means by a "physical object" not some common sense object which is directly observable, but a postulated entity which behaves according to the rules prescribed by the three laws of motion which appear at the beginning of Newton's *Principia*. Similarly, the word "electron" is a fundamentally different concept and refers to a radically different entity in quantum mechanics from what it did in the electro-magnetic theory of Lorentz. This happens because it is a concept by postulation rather than a concept by inspection, and the postulates of quantum mechanics are different from those of Lorentz' theory.

Consequently, one cannot understand the meaning of scientific concepts in the second stage of development of a scientific theory by purely observable and inductive means. Concepts by postulation simply do not refer to anything directly apprehendable. Electrons, protein molecules, and electromagnetic waves are not immediately seen. It is not merely that the classification of such entities involves theoretical factors, after the manner of natural history objects, but also that these scientific objects themselves are meaningless apart from the postulates of deductive theory, and cannot be clearly designated as to their properties or experimentally confirmed as to their existence and causal significance except by recourse to the theoretical and formal, as well as the empirical and inductive, methods of science. This is the reason why modern physics has divided its scientists into two groups, termed "theoretical physicists", whose business it is to master the theoretical and formal, deductive scientific methods for constructing and rigorously developing postulated theory, and "experimental physicists", whose task it is to continue the accumulation of natural history data and to use it to put the logical consequences of the theoretical physicists' deductive theory to an experimental test. It is not an accident that theoretical physicists like Planck, Einstein, and Dirac have played as important a part in the dramatic discoveries of contemporary physics as experimental physicists like Millikan, Bridgeman, and Compton. When an empirical science passes to the second stage of its development and introduces concepts by postulation, concern with theory and deductive, formal, logical methods becomes a scientific necessity.

Since all the major theories of chemistry and physics to which biologists are now appealing are formulated in terms of concepts by postulation, the elucidation of contemporary biological organization is not possible without a clear understanding of the scientific method for formulating and verifying deductive theory which uses such concepts.

B. *The Character of Deductively-formulated Scientific Theory Using Concepts by Postulation*

Since we are concerned with the empirical science of biology, we shall analyze a deductive theory in terms of empirical propositions referring to an empirical subject matter. Were we expounding a deductive theory in pure mathematics, propositional functions rather than propositions would have to be introduced.

Any empirical scientific theory is a body of empirical propositions. An empirical proposition is an expression of which it is significant to predicate truth or falsity.

The propositions of any empirical scientific theory fall into two groups, termed "postulates" and "theorems." The propositions termed postulates are related to those termed theorems by the formal logical relation of formal implication. Given the postulates, the theorems can be derived by nothing but the rules of formal logic. In short, the theorems can be deduced from the postulates; hence, the name "deductively-formulated theory." The postulates of a deductively-formulated theory are those propositions which are assumed in the theory in question as logically unprovable and which are sufficient to enable one to prove, *i. e.*, to logically deduce, the theorems. The theorems of a deductively-formulated theory are all the empirical propositions in the theory other than the postulates. They have the additional essential property of being provable in terms of the postulates. Thus, if one assumes the postulates, formal logic requires one to accept the theorems. This is quite independent of the empirical, as opposed to the formal logical question, of the truth or falsity of the postulates.

Examples of deductively-formulated theories are Euclid's geometry and Newton's mechanics. Given the postulates of Euclid's geometry, all the propositions of Euclid follow necessarily on logical grounds. This is true independently of the empirical question whether the geometry of our universe is Euclidian or non-Euclidian. Similarly, given the postulates of Newton's mechanics, all the propositions in Newton's *Principia* can be proved logically. This necessary connection between the postulates and theorems in Newton's mechanics is just as true today, after the replacement of Newton's postulates by Einstein's, as it was before Einstein's more deductively fertile and empirically accurate theory was discovered.

A postulate or a theorem, *i. e.*, any proposition, in any empirical scientific theory is a collection of concepts. Just as the propositions of a theory fall into two groups, termed postulates and theorems, so its concepts fall into two groups, termed "primitive" or "undefined concepts" and "defined concepts." The defined concepts are derivable from the primitive concepts by the method of definition. Any scien-

tific theory involves primitive or undefined concepts. This follows necessarily because definition involves nothing more than the statement of certain concepts in terms of others. The attempt to define all concepts in a theory, therefore, either sends one off in an infinite regress or leads one in a vicious circle. In a carefully formulated deductive theory the postulates are stated solely in terms of primitive concepts, together with logical constants. A logical constant differs from a primitive concept of an empirical scientific theory in that the primitive concepts hold only for the empirical science in question, whereas logical constants are concepts which are necessarily involved in the expression and formulation of any deductive theory whatever.

The above analysis of a deductive theory makes it clear that the heart of any such theory is specified when the primitive concepts and the postulates are indicated. Given these, all other concepts in the theory can be obtained by the method of definition, and all other propositions by the method of formal implication.

In considering any empirical theory the relation of proof should be clearly distinguished from the relation of truth. Truth is an empirical relation, quite independent of formal logic, between a proposition and immediately apprehended fact. Proof, on the other hand, is a formal logical relation between one proposition and another—that relation, namely, which prescribes when a proposition is a theorem for a given set of postulates. It is to be remembered that a theorem is a proposition which can be deduced from the postulates.

The terming of the basic concepts of a deductive theory as primitive or undefined does not mean that these concepts are meaningless. It entails merely that their meaning is not given by definition. It is designated, instead, by postulation. The meaning of a primitive concept in a deductive theory is prescribed for it by the relations in which it is joined to other primitive concepts of the theory by the postulates. More precisely stated, a primitive concept gains its meaning syntactically, rather than by definition or denotation. This is what we meant when we said earlier in this paper that the meaning of a concept by postulation is prescribed for it by the postulates of the deductive theory in which it occurs. Since one can prescribe for primitive concepts any possible relations to other primitive concepts which the imagination of the empirical scientist, creative philosopher, or the pure mathematician can conceive, quite independently of whether such conceivable entities are directly observable, it is possible, as Professor Dirac suggested, to construct scientific theories in terms of scientific elements and structure quite different from anything which the empirical scientist directly observes. In this manner and by this procedure, the scientific objects, such as electrons, hydrogen ions, wave lengths, protein molecules, etc., of physics and chemistry have arisen.

Since empirical science is concerned not merely with conceivable, but also with empirically-verified theory, the question immediately arises concerning how deductively-formulated theories using such concepts by postulation referring to such unobservable entities can be empirically confirmed or repudiated.

C. *The Method for Empirically Confirming Deductive Theory Formulated in Terms of Concepts by Postulation.*

One consideration with respect to concepts by postulation must be noted. Their definition informs us that a concept by postulation is one the meaning of which is given solely by the postulates of the deductive theory in which it occurs. It follows from this that the concepts in the theorems of such a deductive theory are concepts by postulation also. For we have noted that all concepts in the theorems are either the primitive concepts of the postulates or concepts which reduce to them by definition, these being the only concepts in any deductive theory. Hence, since the concepts in the postulates are concepts by postulation, the concepts in the theorems have to be also.

This consideration lays the basis for a more precise understanding of our present question: How can scientific theory, constructed in terms of concepts by postulation referring to unobservable objects, be empirically confirmed or repudiated?

The usual answer proceeds as follows: Scientific theories which postulate unobservable scientific objects and structures, can none the less be verified by deducing from the postulates of such theories, theorems referring to factors which can be directly observed. Unfortunately this amount of the scientific method will not do. If the theorems are to refer to directly observable factors, their concepts will by definition be concepts by inspection. But we have just shown that in a deductive theory whose postulates are composed of concepts by postulation, the concepts in its theorems must be concepts by postulation also. Consequently, the theorems can refer no more to observable factors than do the concepts of the postulates.

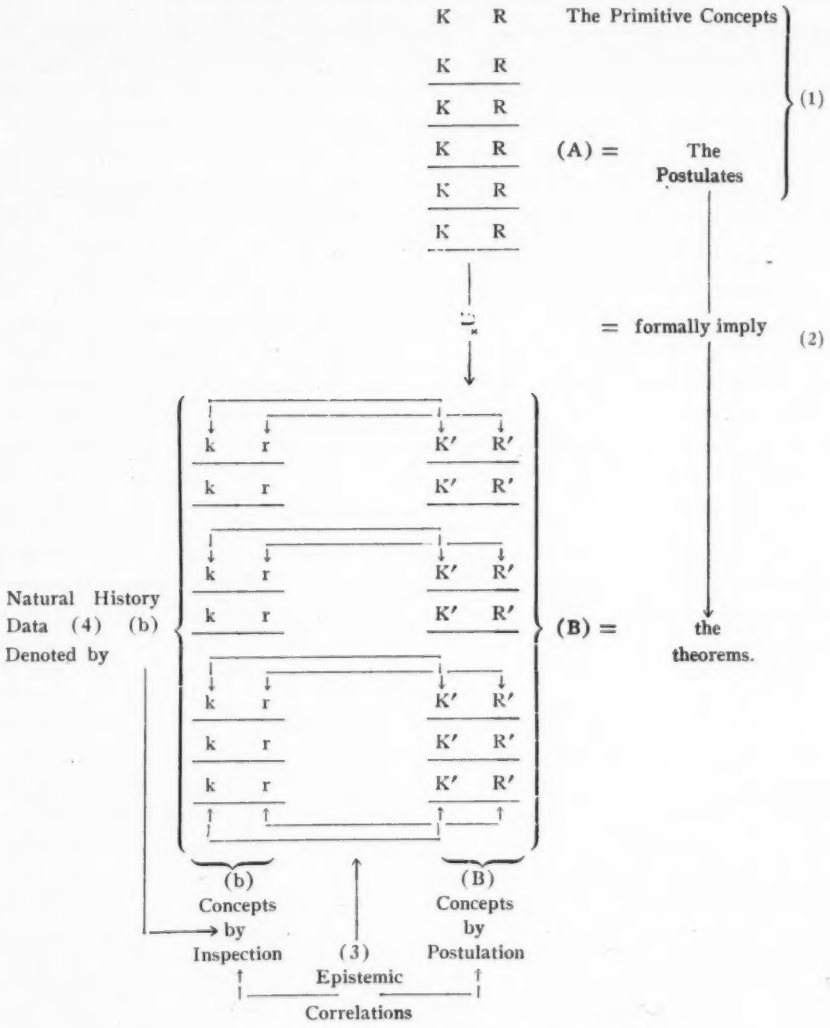
Also, since formal logic, which takes one from the postulates to the theorems, has the power to function only in the world of discourse of the primitive concepts and postulates with which it starts, no manipulation by formal logic can take one from postulates formulated in terms of concepts by postulation to theorems expressed in terms of concepts by inspection. It is one of the merits of formal logic that it never gives one in the conclusion more than is necessarily implicit in the premises. The theory, therefore, that one can verify scientific postulates referring to unobservable entities by deducing from them theorems denoting directly observable factors is untenable. It rests upon a failure to dis-

tinguish concepts by postulation from concepts by inspection, and a consequent muddling of two distinct worlds of discourse.

It appears, therefore, that another factor, previously overlooked, is involved in the empirical verification of deductive scientific theories formulated in terms of concepts by postulation. This factor must be a relation correlating the concepts by postulation in the theorems of the deductively-formulated theory of the theoretical scientist with the directly inspectable data denoted by the concepts by inspection of the natural historian and experimental scientist. It appears, therefore, that the method for experimentally testing deductive theories using concepts by postulation, such as those of physics and chemistry, is much more complicated than has been usually supposed. It involves four major factors: (1) the specification of the primitive concepts and the postulates of the deductively-formulated theory using only concepts by postulation; (2) the derivation of the defined concepts and deduced theorems of the theory by the logical methods of definition and formal implication, giving rise also only to concepts by postulation in the theorems; (3) the specification of relations, which elsewhere I have termed epistemic correlations, (1940) joining systematically and unambiguously the concepts by postulation of the deduced theorems to the concepts by inspection which denote the directly observable natural history materials; (4) the direct, controlled, empirical observation of the prescribed natural history data.

In a recent paper in *Science,* Professor Einstein (1940) writes as follows: "Science is the attempt to make the chaotic diversity of our sense-experience correspond to a logically uniform system of thought. In this system single experiences must be correlated with the theoretic structure in such a way that the resulting coordination is unique and convincing." The important thing to note in this designation of science is the word "correlated." Professor Einstein is saying that the concepts by postulation of the "logically uniform system" of the theoretical physicist are not identified with the immediately apprehended "sense experience(s)" denoted by concepts by inspection of the empirical scientist, but are, instead, joined by what we have termed epistemic correlations. The correlation of 'blue' in the sense of the seen colour to 'blue' in the sense of the wave in electro-magnetic propagations is an example of an epistemic correlation.

Epistemic correlations are not to be confused with the usual statistical correlations of science. The latter always relate items in the same world of discourse, *i. e.,* either they relate one factor designated by concepts by postulation to other factors designated by concepts by postulation, or they relate an inspectable item denoted by a concept by inspection to other inspectable items denoted by the same type of concept.



——— = propositions
———

K R = Primitive Concepts
K′ R′ = Defined Concepts
K′ K_1 = Entities
R R_1 = Relations
KK′RR′ = Concepts by Postulation
kk′rr′ = Concepts by Inspection
$\supset_s$ = the logical relation of formal implication
K′R′ = DfKR = K′R′ is obtained from KR by the method of definition.

The Fourfold Process of Verification
(1) Designation of K, R, and (A).
(2) Deduction of (B) from (A) by $\supset_s$.
(3) Specification of epistemic correlations joining K′R′ to kr.
(4) Inductive Inspection of kr.

Epistemic correlations, on the other hand, always relate a postulated factor designated by a concept by postulation to an inspectable datum denoted by a concept by inspection. It is precisely because they join factors given by two different ways of knowing that we term them "epistemic."

Our analysis of a deductive scientific theory using concepts by postulation and the four stages in its empirical confirmation may now be summarized in the following diagram, code, and definitions.

D. *The Indecisiveness of This Method as Thus Indicated.*

An examination of this diagram and the fourfold process of varification which it exhibits indicates that a certain logic is involved. Let (A) represent the primitive concepts and postulates of the deductive theory which designate the unobserved entities and structures of the scientific theory. Let (B) designate the deduced theorems together with the epistemic correlation joining the concepts by postulation of these theorems to their corresponding concepts by inspection referring to directly inspectable items. It is to be noted that upon this basis a theory is said to be shown to be false when the epistemic, inspectable correlates of its deductive consequences differ from what is naturally or experimentally observed, and that the theory is said to be confirmed when these inspected correlates of the deduced theorems are naturally or experimentally observed. The logic of these two cases is as follows: (1) If A then B; B is not the case; therefore A is not the case. (2) If A then B; B is the case; therefore A is the case.

The logic of these two arguments has been fully and conclusively treated by logicians in their analysis of the hypothetical syllogism (1931). This analysis shows that whereas the argument in the case of the falsification of a scientific theory is formally valid, that in the case of its confirmation is logically invalid, since it commits what logicians term the fallacy of affirming the consequent. We find ourselves, therefore, in this somewhat shocking situation: the method which natural science uses to check the postulationally prescribed theories in its more mature stage of development is absolutely trustworthy when the proposed theory is not confirmed and logically inconclusive when the theory is experimentaliy confirmed.

That this is not a mere concern of pure, formal logicians is indicated by the following consideration which underlies the logicians' analysis. The point is that the experimental inspection of a given set of data designated by B is no proof whatever of the uniqueness of A. There is nothing in the inspectable empirical data themselves which insures that some other quite different theory designated by a set of postulates, C, could not also give rise to the theorems B. In short, it may be the case

not merely that if A then B, B is the case; but also if C then B, B is the case. Einstein has indicated this uncertain status of even our experimentally confirmed scientific theories in the opening paragraph of a paper of his on the work of Clerk Maxwell. Einstein (1934) writes, "The belief in an external world independent of the perceiving subject is the basis of all natural science. Since, however, sense perception only gives information of this external world or of 'physical reality' indirectly, we can only grasp the latter by speculative means. It follows from this that our notions of physical reality can never be final. We must always be ready to change these notions—that is to say, the axiomatic substructure of physics—in order to do justice to perceived facts in the most logically perfect way. Actually a glance at the development of physics shows that it has undergone far-reaching changes in the course of time."

Why, you may ask, in a paper concerned with physics and biology, has it been necessary to labour these logical and methodological details in this manner? There is a very important reason. These considerations show that when a science passes to the second stage of its development, using concepts by postulation and deductively-formulated theory, even its experimentally confirmed theories are never absolutely guaranteed by the factual data. As Einstein has indicated in the last quotation, even our experimentally confirmed scientific theories always assert more than the bare factual data guarantee. This is what the presence of the fallacy of affirming the consequent in the accepted scientific method of mathematical physics indicates. Were the logic of the confirmation of deductive theory as logically impeccable as that of its non-confirmation, experimentally confirmed theories could never turn out to be false with the passage of time. As Einstein puts it, "We can only grasp" the subject matter of physics "by speculative means", and "our notions of" this subject matter "can never be final." This entails that our scientific conclusions always involve a theoretical factor going beyond what the facts logically guarantee and thus makes it absolutely essential in scientific inquiry that we make these theoretical factors explicit and carry on theoretical investigations as well as the accumulation of empirical data. When a science passes to the second, more mature stage of its development, attention upon theory as well as upon data is absolutely essential for scientists if we are to avoid fooling ourselves and are to understand what we are doing.

There is second reason for consideration of these logical and methodological details. A frank facing of the presence of the fallacy of affirming the consequent has caused physical scientists to supplement the method of verification of physical theories as indicated above, by an additional scientific procedure.

E. *The Complete Method of Verifying Deductively-formulated Physical Science*

The key to this more complete method centers in the point previously noted with respect to the fallacy of affirming the consequent. We indicated that the mere experimental confirmation of a deductive theory through its deductive consequences commits this fallacy because it fails to show that the postulates of the theory are unique in their capacity to give rise deductively to the confirmed theorems. This being the case, scientists concluded that one can avoid the danger implicit in accepting a theory upon the basis of an argument which commits a formal, logical fallacy, providing we can supplement the mere experimental confirmation of a given theory, with theoretical investigations going to show that this theory is, to some extent at least, unique in its capacity to give rise deductively to the confirmed consequences. This raises the second question: What method is there, in addition to experimental confirmation, which will indicate the uniqueness of a given set of postulates? The answer to this question is fairly definite and universally accepted by competent scientists. It is as follows: To approximate toward the uniqueness of a deductively-formulated scientific theory it is necessary not merely to have experimental confirmation but also a theoretical investigation of every conceivable theoretical possibility. It is precisely for this reason that the most spectacular advances in modern physics and its most trustworthy theories have depended for their discovery not merely upon the data of the experimentalists but also upon the development and investigations of pure mathematics. For, as Bertrand Russell has said, pure mathematics is the science of all possible worlds and not merely of this higgledy-piggledy job lot of a place. The point is that a scientific theory carries very much more certainty if one can say not merely that its deductively-formulated theorems have been confirmed, but also that after one has investigated all the conceivable or imaginable theoretical possibilities, it is the only one of these possibilities known to date which is confirmed. In fact, this is the only precise way to define a crucial experiment. Such an experiment is not merely one which confirms a given scientific theory; it is, instead, one of a character such that while confirming one theory it repudiates others. Such an experiment is impossible to define without recourse to at least two theoretical possibilities.

This brings us to the final reason for all these technical logical and methodological considerations. The introduction of crucial experiments in order to mitigate the danger of the fallacy of affirming the consequent in the mere experimental confirmation of a scientific theory without the consideration of other theoretical possibilities, means that there can be no trustworthy science, even with experimental confirma-

tion, in the second stage of development of an empirical science unless just as much attention is given by scientists to the consideration of theory and of rival theoretical possibilities and to deductive logic as is given to induction, factual data, and experimentation. The point is that the mere experimental confirmation of a scientific theory through its deductive consequences is not generally regarded by competent scientists as a sufficient criterion of the scientific validity of that theory. One must go further and show as far as is possible that the theory in question is the only one which is capable, through its deductive consequences, of taking care of the natural history data. This is what Professor Einstein meant when, in the designation of science, which we first quoted, he wrote that the "single experiences must be correlated with the theoretic structure in such a way that the resulting coordination is *unique* . . . . . ." Only by formulating rival theories, learning to think deductively with respect to them by pressing them to their deductive consequences—in short, only by emphasis upon theory and deductive logic as well as upon facts and experimentation can the uniqueness of an experimentally confirmed scientific theory be established.

If these considerations are appreciated, we are now prepared to understand the scientific importance of the following investigation of the rival theories of biological organization. We are also equipped with the precise understanding of the scientific method for carrying on such an investigation.

The results of our analysis of the method of the mathematical and physical sciences may now be summarized. Any science in its normal development passes through two stages—the first which we term the natural history stage, the second, that of postulationally prescribed theory. To each of these two stages there belongs a definite type of scientific concept. The type of concept for the natural history stage we term a concept by inspection; that for the postulationally prescribed stage a concept by postulation. A concept by inspection is one the complete meaning of which is given by something immediately apprehended. A concept by postulation is one the meaning of which is prescribed for it by the postulates of the deductive theory in which it occurs. Since it is possible to postulate entities and structures quite different from any immediately observed, science is able to introduce, by recourse to concepts by postulation, unobserved entities and structures into its scientific theories. The existence of such scientific objects is none the less verifiable. The method for this verification is fourfold. The postulated system is prescribed by the postulates of the theory which designates it. From these postulates, by formal logic, theorems are deduced. Between the concepts by postulation in the deduced theorems and concepts by inspection denoting immediately apprehend-

able natural history data, epistemic correlations are set up. When the directly observable epistemic correlates of the deduced theorems of the postulationally prescribed theory are naturally or experimentally observed, the theory is confirmed. If they are not observed or the inspected data are contrary to what is called for, the theory is rejected. In the case of confirmation, a theoretical investigation of alternative hypotheses is pursued, with the purpose of showing as far as is possible not merely that a given theory is confirmed by the data, but also that it is the only theory which is so confirmed.

In the light of these considerations, our task in determining an adequate theory of biological organization is the following one: to find a postulated theory designating a system of related entities which will correlate uniquely with the directly apprehended biological organization exhibited in the natural history data. Let us now examine certain rival theories of biological organization from this standpoint.

III
BIOLOGICAL ORGANIZATION AND THE DEDUCTIVE THEORIES OF PHYSICS

Our account of the normal development of an empirical science would lead us to expect two major types of biological theory—one formulated in terms of natural history data using concepts by inspection; the other appealing to unobserved objects and principles, most likely those of physics and chemistry, using concepts by postulation. This is the case.

A. *The Natural History Theory of Biology*

The major natural history theory of biological organization is that formulated by Aristotle. In modern biology it carries the name of Vitalism. The essential point in this theory is that the concepts of biology are unique and irreducible, in part or whole, to those of any other science. From the standpoint of natural history this is the case. Observed, living creatures are different from observed, inorganic objects. Thus it is natural, in the natural history stage of development of biology, that the irreducibility of this science should be emphasized.

Also, living creatures as immediately observed do exhibit a persistence of form or organization through the influx or outgo of material. This makes it natural to conceive of the living organism as a combination of two main factors: its observed material and its observed form. When one observes also, in the developing embryo, a distribution and rearrangement of the material, so that a given form is produced, it is quite in accord with what one observes to say that the form as well as the matter is an irreducible, causal factor determining the final, mature system. Thus the Aristotelian foundation of science

with its repudiation of any concepts in science or philosophy except those first given through the senses and with its doctrine of both material and formal causes becomes a natural theory for biologists to hold. The great merit of this theory is that it does provide a reasonable solution of the biological problem of organization. If there are irreducible formal factors as well as the material factors, we are then able to understand why there is a persistence of form through a flux of materials and why the matter in the developing embryo arranges itself so that a definite form is actualized.

None the less, this theory, plausible as it seems in the light of the natural history data, runs into certain difficulties. In the first place, all organisms die. Death means a breakdown of directly observed organization. This points toward the conclusion that the organization is not an ultimate, irreducible factor, but something that can come and go. Thus organization seems to point beyond itself to some other more ultimate factors as its own condition. Secondly, there is the modification of species and the origin of new species emphasized by Darwin. This discovery emphasizes the non-irreducibility of form for the species which death emphasizes for the individual. Finally, there has been a third and even more conclusive consideration. Biologists have found for their science what Dirac, in our previous quotation from him, emphasized for physics: a more consistent, adequate conception of the natural history data in biology has been attained by giving up the attempt to understand this data in terms of concepts deriving from directly inspectable items, and by having recourse to the postulated entities of physics and chemistry. The other papers of this symposium give an eloquent demonstration of this point. In short, biologists have come to find that the concepts by postulation of physics and chemistry are more fertile in understanding their own subject matter than the concepts by inspection of an autonomous theory of natural history biology.

Before turning to the consideration of these physical and chemical theories of biological organization, one danger must be noted. In such a transition period in biology from the natural history stage with its concepts by inspection to the stage of postulationally prescribed theory, with its concepts by postulation, it cannot be too strongly emphasized that these two types of concepts belong to two fundamentally different worlds of discourse. Consequently, biological information coming in terms of one type of concept cannot be added together with biological information in terms of the other type of concept to produce anything more than nonsense.

Permit me to make this point more clear with a concrete example. As has been suggested, and as has been emphasized by all leading modern biologists since Claude Bernard, the fundamental problem of

modern biology is that of organization. We know with reasonable certainty what the entities are that constitute a living organism. There is every reason to believe, in the light of the experimental evidence, that these entities are those of physics and chemistry. But when this is admitted, the major problem still remains concerning whether the persistent organization of a living system can reduce to physical and chemical principles also. It is reasonable to say that this problem has not yet been solved. In such a situation it is very easy to attempt to solve the problem by introducing the physical and chemical elements to take care of the constituents of a living organism and adding on the intuited biological organization from the natural history approach to biology to take care of the organization of these constituents. In fact, this is precisely what Hans Driesch and J. S. Haldane have proposed. It must be emphasized, however, that such a thesis is not a scientific theory but nonsense, since it combines, as if in a single world of discourse, the intuited datum of organization which is a concept by inspection, with the entities of physics and chemistry, which are concepts by postulation. This constitutes a muddling of two distinct worlds of discourse.

In the situation in which we find ourselves there are but two alternatives. Either we develop a strictly natural history theory of biology, using only concepts by inspection, or else we have recourse to the entities of physics and chemistry which are joined, as Einstein has indicated, to what we immediately inspect in the natural history stage, by what we have termed epistemic correlations, in which case we must also have concepts by postulation to take care of the organization of these entities.

Since biologists have unequivocally decided to appeal to the entites of physics and chemistry, our task, therefore, is to find a postulated structure provided by physical and chemical theory which can be epistemically correlated with the immediately-observed organization given in the natural history data.

Let us turn, then, to the major theories of chemical and physical science and examine them with respect to the relatedness which their postulates prescribe, to determine whether any one of them provides for a structure which can be epistemically correlated with the natural history data concerning biological organization.

B. *Three Physical Theories of Biological Organization.*

There are three such theories. We shall call them the chemical theory of life, the thermo-dynamical theory of life, and the electrodynamic theory of life respectively.

It is to be emphasized that all three of these theories are implicit in

the chemical factors which have already been experimentally demonstrated to be present in living organisms. Certainly chemical elements involve all the chemical relations which the theory of chemistry prescribes. Unfortunately, the concepts and theory of chemistry have not been formulated deductively with the precision and rigor of physical science. It will be necessary, therefore, for us in considering the chemical theory of life and its deductive consequences with respect to biological organization, to develop this theory somewhat step by step in terms of certain piecemeal experimental findings. In this connection, we shall draw very heavily on the work of Lavoisier.

But chemical elements in their interactions also obey the laws of energy. Consequently, if living organisms involve chemical constituents, the structure of these systems is subject to the laws and postulates of thermo-dynamics. Hence, we have at our service, in attempting to understand biological organization, not merely the laws and information of chemical theory and analysis, but also the deductive consequences of the science of thermo-dynamics.

In addition, chemical entities are known to be constituted of electrical components. Life depends for its very existence upon energy radiated to it upon the earth from the sun. This radiation is an electromagnetic phenomenon. Consequently, living organisms depend for their very existence upon electro-magnetics. This means that not merely the postulates of chemistry and thermo-dynamics, but also those of electro-dynamics are available to provide a postulational structure which may or may not uniquely correlate epistemically with the observed organization of living creatures.

1. *The Chemical Theory of Life.*

Chemistry postulates a collection of atoms, some ninety-two in number, each with unique properties. These atoms enter into combination according to certain proportions and pass out of combination. Thus the emphasis in chemistry is more on the constancy of the entities than on the constancy of the relatedness. This would lead us to expect that the chemical theory of life would be quite effective in accounting for the constituents of living organisms, but somewhat ambiguous in providing an adequate account of the persistence of biological organization. This expectation is not far from the case. Claude Bernard certainly had an appreciation of the tremendous rôle of chemical constituents and processes in living organisms, yet he was equally well aware that the problem of organization remained a problem under this theory. Nevertheless, there are certain findings arising from a chemical approach to biological processes which must be kept in mind and which are absolutely essential for an understanding of biological

organization. No one has made them more evident than the founder of modern physiological chemistry, Lavoisier.

It was Lavoisier who demonstrated that a living system, when viewed chemically, cannot be conceived as the mere collection of chemical compounds within its visible bodily surface. His investigations upon metabolism and respiration showed that the very existence and persistence of a living system depends upon the continuous chemical decomposition of the food materials taken into the organism's body. It is not by finding compounds which hold themselves fixedly together by their chemical bonds, after the manner of the inorganic stone, that the persistence of the living organism has been achieved, but by a continuous breaking down and reorganization and reassembling of chemical constituents. Moreover, this breaking down of carbohydrates which occurs in the animal body in metabolism is impossible without a continuous supply of oxygen. This oxygen has its basis not inside the organism but in the tension of the atmosphere of the earth's surface without. Furthermore, if the carbon dioxide released by the oxidation of the carbohydrates in metabolism were not removed from the organism into the surrounding environment, death would ensue, even though oxidation occurred. Thus the picture we must have of a living organism is not that of a set of local protein molecules with a certain constant structure, whether that structure be conceived of the chain or the pattern type. These molecules constitute but one of a tremendous number of chemical elements not merely within but without the body organism, all of which are in continuous flux and interaction. It is in a most complicated relationship between chemical materials external and internal to the body surface of the organism in which every term in the relationship is in continuous motion and chemical transformation that we must find the concept of the living organism when viewed from the standpoint of the investigations of bio-chemistry.

This is the organism as we find it in the light of chemical analysis. How does it compare with what we would expect upon the basis of the postulates of chemical theory? Curiously enough, the findings are in a way the direct opposite of what chemical theory alone would lead one to expect. According to the theory of atoms upon which chemistry is founded, the persistence should be in the entities, and not in the structure. Yet what we actually find is the reverse. The chemical constituents of the living organism are in continuous motion and flux, and it is the relatedness which persists, or at least changes itself slowly in comparison with the flux of the entities in and out of the relational structure. Dr. Rudolf Schoenheimer's investigations, reported earlier in this symposium, of the behavior of protein molecules in the living organism as exhibited with the technique of "tagged atoms" confirms this point in a spectacular way. These investigations show that the pro-

tein molecule must be continuously built up as constituents are passing out of it and replaced by others continuously. Not even the individual protein molecule persists because of the chemical bonds which hold its constituents together.

These considerations show that while the chemical theory of life is experimentally confirmed and even a uniquely confirmed theory of the constituents of biological organization, it provides, at least in the present stage of its application, very little basis for an understanding of the organization of living things. This does not mean that the theory is false; it means merely that it provides an account of part but not all the problem of organization.

Dr. Schoenheimer's investigations have a second significance. His findings are precisely what one would expect if one conceived of a living organism from the standpoint of the thermo-dynamical theory of life.

2. *The Thermo-dynamical Theory of Life.*

The science of thermo-dynamics informs us that any system which is not in equilibrium in the technical thermo-dynamical sense of this term, will automatically break down, because of the operation of the second law of thermo-dynamics, unless energy is provided for that system from outside. All competent students of living organisms from the standpoint of thermo-dynamics are in agreement upon the fact that living organisms are not in equilibrium in this thermo-dynamical sense. In fact, when a living system passes to a state of maximum entropy, or in other words to a state of thermo-dynamical equilibrium, death takes place. From this it follows that the organization of living systems can only persist if energy comes into them from outside.

This is but another way of saying that the physical-chemical structure of living organisms cannot persist by itself, but must be continuously maintained by energy supplied in the form of food or radiation from without. This is precisely what Dr. Schoenheimer's investigations indicate for even so small a structure as the protein molecule. Left to itself, it would disintegrate. Even to persist, it must have new parts continuously put into it. For this work must be done.

It is because living organisms are not in thermo-dynamical equilibrium, and hence are continuously dependent for their maintenance upon energy from without, that animals depend upon plants directly or indirectly for their food and energy supply, and that plants in turn depend upon energy from the sun. These thermo-dynamical considerations make it evident that if we are to attain a correct physical-chemical theory of the living organism, we must think about it not merely in terms of the relationship between atoms within the organism and those

in the earth's atmosphere and on the earth's surface, but also in terms of the energy relationship and temperature difference between the earth and the sun.

Because of the evolution of living organisms from systems exhibiting relatively simple organization and differentiation to species which become more and more complicated and heterogeneous in their organization, certain students have concluded that the processes of biological growth and evolution are incompatible with the second law of thermo-dynamics. The reason for this merits our consideration and requires a more detailed analysis of the meaning of the first two laws of thermo-dynamics.

The fundamental concept of thermo-dynamics is energy. Energy may be defined roughly as the capacity to do work. The first law asserts that the total amount of energy in any isolated system remains constant.

This total amount of energy divides into two parts. One part is in a form available to do work on organized systems; the other part termed dissipated energy, is in a form unavailable to do work on organized systems. The second law of thermo-dynamics asserts that in an isolated system the amount of energy in this dissipated form unavailable for work on an organized system continuously increases. The dissipated energy unavailable for work is termed entropy. Thus the second law takes on the form stated by Clausius: the entropy of the universe tends toward the maximum.

Since it takes more energy in a form available to do work to maintain a complicated and highly organized biological system than to organize and maintain a simpler system, and since biological evolution does proceed from a relatively homogeneous type of organism to a more and more heterogeneous species, it would seem, upon first sight, that biological systems go directly counter to the second law of thermo-dynamics, by proceeding from a state involving a lesser amount of energy in a form capable of doing work to a state involving a greater amount of such energy. Certain students of thermo-dynamics in its relation to biological evolution, notably Sir James Jeans, have reached this conclusion .Later, Professor F. G. Donnan (1934) pointed out , quite correctly that there is no contradiction between biological evolution towards systems requiring more and more energy in a form available for work and the second law of thermo-dynamics.

One reason, in the opinion of this writer, more important than that given by Professor Donnan, is that the second law does not assert that the entropy of a system can never decrease with time. It asserts merely that this can never be the case in an isolated system. The second law of thermo-dynamics is quite compatible with a decrease of entropy in

the evolution of a given system, providing that energy in a form available for work is brought into that system from outside.

This we know to be the case since, as we pointed out previously, animals depend directly or indirectly upon plants for their food and energy supply, and plants in turn directly depend upon energy from the sun.

This means, however, that we cannot expect to find the scientific basis of biological growth and organization solely within the chemical constituents of the animal's body. Growth and organization involve chemical factors within and without the animal body on the earth's surface and in its atmosphere, and these in turn depend upon energy relations with our solar system. Were it not for the temperature difference between the sun and the earth, biological growth, organization, and evolution as we know it, would be impossible on thermo-dynamical grounds. Such considerations must make one exceedingly skeptical of all attempts to find the key to growth and biological organization in some local chemical factor, whether it be auxin or the structure of the protein molecule, important as these items are as one among a tremendous number of variables in the complicated, many-termed chemical, and thermo-dynamical relationship which constitutes the living organism.

There is a sense, however, in which Sir James Jeans as well as Professor Donnan may be correct, with respect to the relation between biological evolution and the second law of thermo-dynamics. Professor Herbert Freundlich raised this consideration at the Salisbury Symposium after the discussion of this paper. Before it could be treated, the discussion at the symposium went off on another topic. The point is this: by an appeal to energy coming to the earth and into biological systems from the sun, one does quite properly reconcile biological evolution with the second law of thermo-dynamics after the manner indicated by Donnan. This presupposes, however, a certain assumption concerning what happens so far as the second law is concerned in the astronomical universe as a whole. To maintain the second law in the face of biological evolution, it is necessary to affirm, for the astronomical universe as a whole, that the decrease in entropy taking place in the evolution of biological systems is more than equalled by a corresponding increase in entropy somewhere else in the universe, so that the sum total of entropy in biological evolution and the rest of the universe tends toward a maximum. This is a topic upon which there is no absolutely conclusive astronomical evidence and one with respect to which there are rival hypotheses. These hypotheses will vary, furthermore, depending upon whether one postulates, as traditional, modern science did, an astronomical universe with infinite extension, or as contemporary astro-

physics is inclined to do, an astronomical universe which is finite (Northrop, 1931).

The important point from thermo-dynamics in its bearing on biological growth and evolution, for our present purposes, however, is that it forces us to conceive of biological systems as a complicated, many-termed relation between chemical entities in continuous motion and flux, both internal and external to the organism's body and surface, this relation between these many moving chemical entities being continuously dependent upon energy coming into the system from a solar source outside.

This conclusion is important for two reasons. First, it reminds us that biological organization is not a static, chemical bond between persistent chemical entities. In short, the persistence of the organization does not have its basis in the persistency of the entities which are the terms in its relatedness. Biological organization, instead, is a relation with certain formal properties, holding between entities continuously passing in and out of this organization. It is, to be precise, the form of the relation between the motion of its parts. Secondly, the thermo-dynamical dependence of biological growth and evolution upon energy from the sun establishes, in the most literal sense of the word, that biological organization has its origin in electro-magnetics as well as in chemistry and thermo-dynamics. This follows because the energy coming into the conglomeration of chemical materials from outside is brought to the earth and to plants upon its surface from the sun by electro-magnetic propagations. In fact, the synthesis of carbohydrates and the resultant storing of solar energy which takes place in plants by the mediation of chlorophyll is an electro- or photo-chemical process. This brings us to a third theory of physical science which must have significance for biology. We term it the electro-dynamic theory of life.

3. *The Electro-dynamic Theory of Life.*

Our consideration of chemical theory and thermo-dynamical theory in relation to biology informs us that the postulates of chemistry are adequate to account for the constituents of organization and that the principles of thermo-dynamics are necessary and consistently able to account for the energy necessary to organize those constituents.

Although these two theories are necessary, they are by no means sufficient. The insufficiency of the chemical theory has been summarized by saying that it could account for the persistence of biological organization only if organization had its basis in the persistence in the organism of the individual entities which are the chemical terms of that organization. That this is not the case is shown by the fact that although the organization persists, the chemical atoms and molecules

within this organization are in continuous motion and flux. The insufficiency of the thermo-dynamical theory as a complete account of biological organization centers in the fact that there is nothing in the theory to prescribe the particular relatedness into which the energy organizes the moving, chemical materials. This can be put in more technical language by saying that there is nothing in thermo-dynamics itself which prescribes at precisely what point in the tendency toward a state of maximum entropy the energy from outside the system compensates that tendency, to produce the steady state, or the state of mean compensated entropy, which is a living organism.

This inadequacy of traditional physical-chemical analyses of biological processes was emphasized in previous sessions of this symposium by Professor E. F. Adolph. He indicated at many points that while we know a great deal about certain chemical constituents, we are very far from having a physical-chemical theory which provides either in its postulates or theorems, anything which can be set in any kind of systematic, epistemic correlation with the actual natural history data of biological organization. What seems to be called for in addition to the concepts by postulation of chemistry and thermo-dynamics, is a postulational theory of physics which will prescribe an irreducible relatedness between moving physio-chemical entities. This is precisely what field physics provides. The electro-dynamic theory of life is merely the conception of living organisms from the standpoint of field physics.

The fundamental postulate of field physics has been stated clearly by Clerk Maxwell, the first scientist to formulate this theory mathematically and deductively. Elsewhere (1938) we have stated this as follows: "In his first famous paper on electro-magnetics, entitled *On Faraday's Lines of Force,* after indicating that Ampere's theory of electric currents assumes 'attracting forces considered as due to the mutual action of particles,' Maxwell writes, 'but we are proceeding upon a different principle, and searching for the explanation of the phenomenon, not in the currents alone, but also in the surrounding medium.' In other words, instead of beginning with the particles related by forces acting instantaneously at a distance, and attempting to define the field and its organization by mere compounding, as does particle physics, Maxwell is forced in order to account for the existence of light and other electro-magnetic propagations in the universe, to begin with the field with its relatedness as in fact continuous and irreducible and to derive in part at least the location and motion of the charged particles in the electric current.

"Furthermore, form as well as continuity is assumed for the field. Maxwell writes (l. c. p. 205): 'The distribution of the currents due to these field forces depends upon the form and arrangement of the conducting medium.' In his final paper on electro-magnetics, containing

his famous equations, he adds 'this medium must be so connected that the motion of one part depends upon the motion of the rest (l. c. p. 528).' One might think it were a biologist talking about the type of assumption requisite for an adequate theory of biological organization.

"A few pages later, when Maxwell describes his specific procedure, he is even more explicit in his designation of the relatedness of the system as in part an original irreducible causal factor determining the mechanical motions of the parts. We begin, he says, with 'the form of the relation between the motion of the parts,' given in the laws of induction, and 'The second result, which is deduced from this, is the mechancial action between conductors carrying currents (l. c. p. 533).' At last, we have a physical theory which if it applies to a living creature, begins to make sense of the fact that it is not merely a collection of chemicals requiring energy but also an individual which is organized. The relational factor necessary to provide and in part preserve the form of the whole as well as the atomic factor necessary to account for the chemistry of the parts is at hand."

The important point to note is that the introduction of the postulates of field physics with their prescription of an irreducible relatedness has been found to be necessary to account even for phenomena in the inorganic world. Following the tremendous success of Newtonian mechanics with its postulation of mass particles related by forces acting instantaneously at a distance the attempt was made by Ampere and others to formulate electricity, magnetism, and optics in such terms. With electricity and magnetism this attempt was successful, although Faraday had brought forth convincing experimental evidence suggesting that it might be more naturally conceived in terms of a field concept. Before the phenomenon of optics, requiring an undulatory theory of light, particle physics broke down completely. Consequently, when Maxwell developed Faraday's field concept mathematically and deductively, and was able to show that the undulatory theory of light followed from it necessarily, field physics triumphed not merely in optics but also in electricity and magnetism. Since Maxwell's time it has gone on to further triumphs. With Lorentz and Larmor it was generalized, so that it permitted the prediction of the negatively charged electron on theoretical grounds before this postulated scientific object was discovered experimentally by Sir J. J. Thomson. In this connection it must be realized that the electron is not, as so many people suppose, a concept of particle physics, but is instead defined in terms of the assumptions of field physics. With Einstein, these assumptions, in his theory of relativity received an even more spectacular generalization and experimental confirmation.

As Einstein has written, "For several decades most physicists clung to the conviction that a mechanical substructure could be found for

Maxwell's theory. But the unsatisfactory results of their efforts led to gradual acceptance of the new field concepts as irreducible fundamentals" (1940). In short, what Maxwell termed "the form of the relation between the motion of the parts" has been accepted by physicists as irreducible.

If physicists have been driven to the postulation of such irreducible relatedness of moving parts to account for inorganic phenomena, is it not all the more reasonable to suppose that this postulate of electromagnetic theory is required for living organisms with their even more obviously persisting relatedness? Guided by reasoning of this kind, my colleague, H. S. Burr, and I, (1935) put forward what we termed the electro-dynamic theory of life—the theory, that is, that an understanding of biological organization is to be found if we conceive it from the standpoint of the postulates of electro-magnetic theory.

If this hypothesis be correct, it follows that electro-metric experimental methods should detect systematic, organic, electrical properties of living organisms. Guided by this hypothesis, Professor Burr, in conjunction with Drs. Lane and Nims (1936), developed a new experimental apparatus to put this theory to an empirical test. This apparatus has been applied to many different species of living organisms. In every case it has been found that systematic, distributed, potential differences exhibit themselves over any living organism considered as a whole. The details concerning these findings have been published elsewhere (1937). I would merely add here one recent finding by Dr. Burr, the details of which are yet to be published. More than any previous experiment, this one shows that at last in this theory we have the type of epistemic correlation between the postulated structure of physical theory and the directly inspected natural history data of organization which our previous analysis of the scientific method for handling postulational physical theory has shown to be required for any trustworthy scientific solution of the biological problem.

The experiment is as follows: on a fertilized egg of amblystoma in one of its early stages of embryonic development, when no directly inspectable differentiations of the natural history type were observable, Dr. Burr found, with his experimental apparatus, a definite organized pattern of potential differences. Using dyes, he designated an inspectable pattern on the surface of the organism corresponding to the postulated potential differences designated by his apparatus. When the organism was allowed to grow, later, inspectable, natural history differentiations appeared at precisely the locations which his dyes, as guided by his electro-metric readings, had indicated. This suggests quite definitely that electrical differentiation is at the basis of the developing differentiation of the natural history data. It indicates,

also, that the requirements of sound, scientific method for solving the problem of biological organization are satisfied. That is, epistemic correlations have been established between the postulated electrical distinctions of physical theory and the directly observable data of the natural historians' inductive observation of biological organization.

It would appear, therefore, that in the relationship between the fundamental concepts of chemical theory, thermo-dynamical theory, and the electro-magnetic theory of field physics, a scientific theory for the solution of the problem of organization in biology is to be found. Chemical theory provides the postulated entities at the basis of the material constituents of living organisms; thermo-dynamics provides an understanding of their dependence on energy factors from without, and the electro-dynamic theory provides the irreductible relatedness necessary for an understanding of the organization of the constituents as worked upon by the energy.

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